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(54) Title: ORPHAN NUCLEAR RECEPTOR

(57) Abstract

The present invention relates to a novel human orphan nuclear receptor that binds to a cytochrome P–450 monooxygenase (CYP) promoter and that is activated by compounds that induce CYP gene expression. The invention further relates to nucleic acid sequences encoding such a receptor, to methods of making the receptor and to methods of using the receptor and nucleic acid sequences encoding same. The invention also relates to non–human animals transformed to express the human receptor and to methods of using such animals to screen compounds for drug interactions and toxicities.

ORPHAN NUCLEAR RECEPTOR

The present application claims priority from
Provisional Application No. 60/079,593, filed March
27, 1998, the entire contents of that provisional
5   application being incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to a novel human
orphan nuclear receptor that binds to a cytochrome
P-450 monooxygenase (CYP) promoter and that is
10   activated by compounds that induce CYP gene
expression. The invention further relates to
nucleic acid sequences encoding such a receptor, to
methods of making the receptor and to methods of
using the receptor and nucleic acid sequences
15   encoding same. The invention also relates to non-
human animals transformed to express the human
receptor and to methods of using such animals to
screen compounds for drug interactions and
toxicities.

20   BACKGROUND OF THE INVENTION

Members of the cytochrome P-450 (CYP) family of
hemoproteins are critical in the oxidative
metabolism of a wide variety of endogenous
substances and xenobiotics, including various
25   carcinogens and toxins (Nebert et al, Ann. Rev.
Biochem. 56:945-993 (1987)). In man, the CYP3A4
monooxygenase plays a major role in the
biotransformation of drugs due to its abundance in
liver and intestine and its broad substrate

specificity. CYP3A4 catalyzes the metabolism of >60% of all drugs that are in use including steroids, immunosuppressive agents, imidazole antimycotics, and macrolide antibiotics (Maurel, P.

5   in Cytochromes P450: metabolic and toxicological aspects (ed. Ioannides, C.) 241-270 (CRC Press, Inc., Boca Raton, FL, 1996).

Expression of the CYP3A4 gene is markedly induced both *in vivo* and in primary hepatocytes in

10 response to treatment with a variety of compounds. Many of the most efficacious inducers of CYP3A4 expression are commonly used drugs such as the glucocorticoid dexamethasone, the antibiotic rifampicin, the antimycotic clotrimazole, and the

15 hypocholesterolemic agent lovastatin (Maurel, P. in Cytochromes P450: metabolic and toxicological aspects (ed. Ioannides, C.) 241-270 (CRC Press, Inc., Boca Raton, FL, 1996), Guzelian, P.S. in Microsomes and Drug Oxidations (eds. Miners, J.O.,

20 Birkett, D.J., Drew, R. & McManus, M.) 148-155 (Taylor and Francis, London, 1988). The inducibility of CYP3A4 expression levels coupled with the broad substrate specificity of the CYP3A4 protein represent the basis for many drug

25 interactions in patients undergoing combination drug therapy. While attempts have been made to develop *in vivo* and *in vitro* assays with which to profile the effects of compounds on CYP3A expression levels, these efforts have been hampered by species-specific

30 effects that have limited the utility of using animals and their tissues for testing purposes. Thus, analysis of the effects of new compounds on

CYP3A4 gene expression has been largely restricted to laborious assays involving human liver tissue.

Recently, efforts have been directed at understanding the molecular basis for the induction
5   of CYP3A4 gene expression. The CYP3A4 promoter has been cloned and a 20 bp region residing approximately 150 bp upstream of the transcription initiation site shown to confer responsiveness to dexamethasone and rifampicin (Hashimoto et al, Eur.
10   J. Biochem. 218:585-595 (1993), Barwick et al, Molec. Pharmacol. 50:10-16 (1996)). This region contains two copies of the AG(G/T)TCA motif recognized by members of the nuclear receptor superfamily, suggesting that a nuclear receptor
15   might be responsible for mediating at least some of the effects of the chemical inducers of CYP3A4 expression. However, prior to the present invention, proteins that bind to this response element had not been characterized.
20   The present invention is based on the identification of a novel orphan nuclear receptor that binds to a response element in the CYP3A4 promoter and that is activated by a range of compounds known to induce CYP3A4 expression. The
25   identification of this receptor makes possible assays that can be used to establish whether drugs will interact *in vivo*.

SUMMARY OF THE INVENTION

The present invention relates to a novel human
30   orphan nuclear receptor, designated the human pregnane X receptor (hPXR), that binds to a CYP

promoter, for example, the rifampicin/dexamethasone
response element in the cytochrome P-450
monooxygenase 3A4 (CYP3A4) promoter. The receptor
is activated to modulate transcription of a CYP
5 (e.g., CYP3A4) gene. The present invention further
relates to nucleic acids encoding hPXR, including
expression vectors that can be used to effect
expression of the receptor in host cells. The
invention also relates to host cells transformed
10 with such expression vectors and to methods of using
the receptor and receptor encoding sequences in
assays designed to screen compounds (e.g., drugs)
for their ability to modulate CYP (e.g., CYP3A4)
gene expression. The invention also relates to non-
15 human animals transformed to express the human
receptor and to methods of using same in drug
screens.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D. Molecular cloning of hPXR.
20 (Fig. 1 A) Nucleotide (SEQ ID NO:13) and predicted
amino acid (SEQ ID NO:14) sequences of hPXR.
(Fig. 1B) Amino acid sequence comparison between
hPXR, mPXR1, Xenopus orphan nuclear receptor 1
(xONR1) (Smith et al, Nucl. Acids Res. 22:66-71
25 (1994)), and the human vitamin D receptor (hVDR).
Numbers indicate percent amino acid identity in the
DBDs and LBDs. (Fig. 1C) The hPXR clone encodes a
functional nuclear receptor. Transfection assays
were performed with a pSG5-hPXR expression vector
30 containing the wild-type 5' region of the hPXR cDNA
and a reporter plasmid containing four copies of the

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CYP3A1 DR3 PXRE. Cells were treated with vehicle alone (0.1% DMSO) or 10 µM of dexamethasone-t-butylacetate. Cell extracts were subsequently assayed for CAT activity. Data points represent the
5   mean of assays performed in duplicate. (Fig. 1D) Translation of the full-length hPXR initiates at a non-AUG codon. *In vitro* transcription and translation were performed with the pSG5-hPXR expression vector containing the wild-type 5' region
10   of the hPXR cDNA or pSG5-hPXR AUG, in which the CUG codon at nucleotide positions 304-306 was modified to AUG. The 50 kD product synthesized when either template was used is indicated by the open arrow and the asterisk. Two shorter products which are likely
15   to represent translation initiation at methionine-56 and methionine-69 within the DBD are indicated by closed arrows. A longer translation product present at low levels is indicated by the bent arrow. Size markers (in kD) are indicated at left.

20   Figure 2. Northern blot analysis of hPXR expression pattern in adult tissues (left to right, heart (1), brain (2), placenta (3), lung (4), liver (5), skeletal muscle (6), kidney (7), pancreas (8), spleen (9), thymus (10), prostate (11), testis (12),
25   ovary (13), small intestine (14), colon (15), PBL (16). RNA size markers (in kb) are indicated at left.

      Figures 3A-3C. hPXR activates transcription through an IR6 element in the CYP3A4 promoter.
30   (Fig. 3A) CV-1 cells were cotransfected with the (IR6)$_3$-tk-CAT reporter plasmid in either the absence

(-) or presence (+) of the pSG5-hPXR ATG expression
plasmid and treated with vehicle alone (open bars)
or 10 μM dexamethasone-t-butylacetate (closed bars).
Cell extracts were subsequently assayed for CAT
5   activity. Data represent the mean of assays
performed in triplicate +/- S.E. (Fig. 3B)
Oligonucleotides used in band shift assays. The
positions of nuclear receptor half-site motifs and
mutations are indicated. (Fig. 3C) Band shift
10 assays were performed with a radiolabeled
oligonucleotide containing the CYP3A4 IR6 PXRE and
hRXR and either hPXR (top panel) or mPXR1 (bottom
panel). Unlabeled competitor oligonucleotides were
added at a 10-fold or 50-fold molar excess as
15 indicated.

    Figures 4A-4C. hPXR is activated by
structurally-distinct inducers of CYP3A4 gene
expression. (Fig. 4A) CV-1 cells were transfected
with the pSG5-hPXR ATG or pSG5-mPXR1 expression
20 plasmids and the (IR6)$_3$-tk-CAT reporter (left and
middle panels, respectively), or the RS-hGR
expression plasmid (Giguere et al, Cell 46:645-652
(1986)) and a reporter containing two copies of a
consensus glucocorticoid response element upstream
25 of tk-CAT (right panel). Cells were treated with
1 μM mevastatin or lovastatin, 100 μM phenobarbital,
or 10 μM of the other compounds. Cell extracts were
subsequently assayed for CAT activity. Data
represent the mean of assays performed in triplicate
30 +/- S.E. (Fig. 4B) Structures of representative
compounds that activate hPXR. (Fig. 4C) CARLA was
performed with bacterially-expressed GST-hPXR or

GST-mPXR1 and [^{35}S]SRC1.14 synthesized *in vitro*.
[^{35}S]SRC1.14 was mixed with either GST-hPXR or GST-
mPXR1 in the presence of vehicle alone (1) (1% DMSO)
or 10 μM of dexamethasone-t-butylacetate (2),

5   rifampicin (3), or clotrimazole (4). [^{35}S]SRC1.14
complexed with GST-hPXR (top panel) or GST-mPXR1
(bottom panel) was precipitated with glutathione-
sepharose beads.

     Figure 5. Reaction scheme for production of
10   [^{3}H]GW-485801.

     Figure 6. Plot of specific binding vs.
concentration of [^{3}H]GW-485801. Kd = 370 nM.

DETAILED DESCRIPTION OF THE INVENTION

     The present invention relates to a novel human
15   nuclear receptor, hPXR. The invention further
relates to nucleic acid sequences encoding hPXR, to
constructs comprising such sequences, to host cells
containing the constructs and to a method of
producing hPXR using such host cells. The invention
20   also relates to non-human animals transformed to
express hPXR. The invention further relates to *in
vivo* and *in vitro* assays that can be used to
identify compounds that induce CYP expression.
While the disclosure that follows makes specific
25   reference to CYP3A4, it should be appreciated that
the details (e.g., methods) provided find
application in connection with other CYP genes as
well.

hPXR is characterized as a protein comprising about 434 amino acids and having a molecular weight of about 49.7 kilodaltons. hPXR binds to a DNA response element in the CYP3A4 promoter as a

5   heterodimer with the 9-cis retinoic acid receptor, RXR. hPXR is activated by compounds known to modulate CYP3A4 expression. The receptor is most abundantly expressed in liver but is also present in colon and small intestine.

10   One embodiment of the receptor of the invention has the amino acid sequence set forth in Figure 1, or an analog thereof (wherein the term analog is intended to indicate a naturally occurring human variant of the Figure 1 sequence), or a fragment

15   thereof, including fragments having at least one functional characteristic of hPXR (e.g., ligand binding or DNA binding). Preferred fragments include portions of the Figure 1 sequence at least 30 consecutive amino acids in length, more

20   preferably, at least 50 consecutive amino acids in length, and most preferably, at least 75 consecutive amino acids in length. Specific fragments include the ligand binding domain (that is, amino acids 141 to 434 of the Figure 1 sequence) and the DNA binding

25   domain (that is, amino acids 41 to 107 of the Figure 1 sequence) as well as the domain that is used for the ligand binding assay described in the Examples that follow (that is, amino acids 130-434 of the Figure 1 sequence). The invention also includes a

30   protein comprising a domain sharing at least 80% amino acid sequence identity with the ligand binding domain of the Figure 1 sequence, more preferably, at

least 85% amino acid sequence identity and, most
preferably, at least 90% or 95%, 96%, 97%, 98% or
99% amino acid sequence identity with the ligand
binding domain of the Figure 1 sequence (% sequence
5   identity being determined, for example, by Basic
Blast (version 2.0) available through the NCBI
website http://www.ncbi.nlm.nih.gov/), and,
advantageously, retaining the function of the
Figure 1 sequence.

10   The receptor of the invention, or fragment
thereof, can bear a detectable label (e.g., a
radioactive or fluorescent label). The receptor, or
receptor fragment, can also be bound to a solid
support, e.g., a glass or plastic particle, a plate,
15   or a filter.

Nucleic acid sequences of the invention include
DNA and RNA sequences encoding hPXR, for example,
hPXR having the amino acid sequence given in
Figure 1, as well as nucleic acid sequences encoding
20   analogs and fragments of the Figure 1 amino acid
sequence as defined above, and nucleic acid
sequences encoding proteins comprising a domain
sharing at least 80% amino acid sequence identify
(more preferably, at least 85%, or at least 90%, or
25   at least 95%, or at least 96%, or at least 97%, or
at least 98% or at least 99%) with the ligand
binding domain of the Figure 1 sequence, as
described above. A specific nucleic acid sequence
of the invention is that shown in Figure 1.

30   The hPXR encoding sequence can be present in a
construct, for example, in an expression construct,
operably linked to a promoter (e.g., the CMV, SV40,

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Taq, T7 or LacO promoter). Such expression
constructs are operative in a cell in culture (e.g.,
yeast, bacteria, insect or mammalian), to express
the encoded hPXR, or fragment thereof. Preferred
5   expression vectors include pGEX, pET, pFASTbacHT and
pSG5.

The invention also relates to cells in culture
(e.g., yeast, bacteria or mammalian (for example,
CV-1, HuH7, HepG2, or CaCo2 cells)) that are
10   transformed with an above-described construct.
Transformation can be effected using any of a
variety of standard techniques. Such cells can be
used in a method of making hPXR (or fragment
thereof) by culturing same under conditions suitable
15   for expression of the polypeptide product.

The invention further relates to chimeric
receptors (or fusion proteins having a receptor
component) (and encoding sequences) comprising at
least a DNA-binding domain or a ligand-binding
20   domain of hPXR, and a non-hPXR derived sequence.
Non-hPXR derived sequences can be selected so as to
be suitable for the purpose to be served by the
chimeric receptor. Examples of such sequences
include glutathione-S-transferase and the DNA
25   binding domain of yeast transcription factor GAL4
and other DNA binding domains, e.g., DNA binding
domains for the estrogen and glucocorticoid
receptors. The chimeric receptor can bear a
detectable label (e.g., a radioactive or fluorescent
30   label). The chimeric receptor can also be bound to
a solid support, e.g., a glass or plastic particle,
a plate or a filter.

A further aspect of the invention relates to *in vitro* (cell-free) and *in vivo* (cell-based) assays that can be used to profile the effects of compounds (e.g. potential new drugs) on CYP3A4 levels. The

5　inducibility of CYP3A4 levels, coupled with the broad substrate specificity of the CYP3A4 enzyme, represent the basis for many drug-drug interactions in patients undergoing multiple drug therapy. Ideally, new drugs would have little or no effect on

10　CYP3A4 expression levels.

The assays of the invention can take any of a variety of forms. As compounds that activate hPXR function as inducers of CYP3A4 gene expression, hPXR binding and activation assays provide efficient

15　means to identify compounds that can be expected to activate CYP3A4.

Binding assays of the invention include cell free assays in which hPXR, or the ligand binding domain thereof (alone or present as a fusion

20　protein), is incubated with a test compound which, advantageously, bears a detectable label (e.g., a radioactive or fluorescent label). The hPXR, or ligand binding domain thereof, free or bound to test compound, is then separated from free test compound

25　using any of a variety of techniques (e.g., using gel filtration chromatography (for example, on Sephadex G50 spin columns) or through capture on a hydroxyapatite resin). The amount of test compound bound to hPXR or ligand binding domain thereof, is

30　then determined (for example, by liquid scintillation counting in the case of radiolabelled test compounds).

An alternative approach for detecting
radiolabeled test compound bound to hPXR, or ligand
binding domain thereof, is a scintillation proximity
assay (SPA). In this assay, a bead (or other
5   particle) is impregnated with scintillant and coated
with a molecule that can capture the hPXR, or ligand
binding domain thereof (e.g., streptavidin-coated
beads can be used to capture biotinylated hPXR
ligand binding domain). Radioactive counts are
10 detected only when the complex of radiolabeled test
compound and the hPXR, or ligand binding domain
thereof, is captured on the surface of the SPA bead,
bringing the radioactive label into sufficient
proximity to the scintillant to emit a signal. This
15 approach has the advantage of not requiring the
separation of free test compound from bound (Nichols
et al, Anal. Biochem. 257:112-119 (1998)).

Assays to determine whether a test compound
interacts with the hPXR ligand binding domain can
20 also be performed via a competition binding assay.
In this assay, hPXR, or ligand binding domain
thereof, is incubated with a compound known to
interact with hPXR, which compound, advantageously,
bears a detectable label (e.g., a radioactive or
25 fluorescent label (see Example 5 - Crabtree
catalysts suitable for use in the synthetic approach
described in Example 5 include those reported by
Chen et al, J. Labelled Compd. Radiopharm. 39:291
(1997) and Crabtree et al, Inorg. Synth. 28:56
30 (1990))). A test compound is added to the reaction
and assayed for its ability to compete with the
labeled compound for binding to hPXR, or ligand

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binding domain thereof. A standard assay format
employing a step to separate free known (labeled)
compound from bound, or an SPA format, can be used
to assess the ability of the test compound to
5   compete.

A further example of a binding assay in
accordance with the invention is based on the
finding that hPXR ligands induce the interaction of
hPXR ligand binding domain with coactivators (e.g.,
10   SRC1, TIF-1, TIF-2 or ACTR, or fragment thereof).
To determine if a test compound activates hPXR, and
thus induces CYP3A4 expression, the ligand binding
domain of hPXR is prepared (e.g., expressed) as a
fusion protein (e.g., with glutathione-S-transferase
15   (GST), a histidine tag or a maltose binding
protein). The fusion protein and coactivator
(either or both advantageously labeled with a
detectable label, e.g., a radiolabel or fluorescent
tag) are incubated in the presence and absence of
20   the test compound and the extent of binding of the
coactivator to the fusion protein determined. The
induction of interaction in the presence of the test
compound is indicative of an hPXR activator.

hPXR activation assays in accordance with the
25   invention can be carried out using full length hPXR
and a reporter system comprising one or more copies
of the DNA binding site recognized by the hPXR
binding domain (see Example 3). Advantageously,
however, the activation assays are conducted using
30   established chimeric receptor systems. For example,
the ligand binding domain of hPXR can be fused to
the DNA binding domain of, for example, yeast

transcription factor GAL4, or that of the estrogen
or glucocorticoid receptor. An expression vector
for the chimera (e.g.,the GAL4-hPXR chimera) can be
transfected into host cells (e.g., CV-1, HuH7, HepG2

5   or CaCo2 cells) together with a reporter construct.
The reporter construct can comprise one or more
(e.g., 5) copies of the DNA binding site recognized
by the binding domain present in the chimera (e.g.,
the GAL4 DNA binding site) driving expression of a

10   reporter gene (e.g., CAT, SPAP or luciferase).
Cells containing the constructs are then treated
with either vehicle alone or vehicle containing test
compound, and the level of expression of the
reporter gene determined. In accordance with this

15   assay, enhancement of expression of the reporter
gene in the presence of the test compound indicates
that the test compound activates hPXR and thus can
function as an inducer of CYP3A4 gene expression.
(See Example 4.)

20   Another format suitable for use in connection
with the present invention is the yeast two-hybrid
assay. This is an established approach to detect
protein-protein interactions that is performed in
yeast. Protein #1, representing the bait, is

25   expressed in yeast as a chimera with a DNA binding
domain (e.g., GAL4). Protein #2, representing the
predator, is expressed in the same yeast cell as a
chimera with a strong transcriptional activation
domain. The interaction of bait and predator

30   results in the activation of a reporter gene (e.g.,
luciferase or β-galactosidase) or the regulation of
a selectable marker (e.g., LEU2 gene). This

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approach can be used as a screen to detect, for
example, ligand-dependent interactions between hPXR1
and other proteins such as coactivator proteins
(e.g., SRC1, TIF1, TIF2, ACTR) or fragments thereof.
5   (Fields et al, Nature 340:245-246 (1989)).

    Still another format is the ligand-induced
complex formation (LIC) assay. This is an approach
to detect ligand-mediated effects on nuclear
receptor-DNA interactions. hPXR (or, minimally, the
10 DNA and ligand binding domains thereof) can be
incubated with its heterodimeric partner RXR in the
presence of DNA representing an established hPXR/RXR
binding site. Test compounds can be assayed for
their ability to either enhance or interfere with
15 binding of the hPXR/RXR heterodimer to DNA (Forman
et al, Proc. Natl. Acad. Sci. USA 94:4312-4317
(1997)).

    Compounds that bind PXR with a suitable pKi,
for example with a pKi > 5, can be screened for
20 selectivity for PXR versus other nuclear receptors
(e.g., RXR) using standard binding assays. A
compound that binds selectively to PXR (that is, has
at least a 10 fold greater affinity for PXR,
preferably, at least a 100 fold greater affinity for
25 PXR, than, for example, the glucocorticoid receptor)
and thereby affects the functional activity of PXR
in a cell (e.g., a cell in culture, a cell present
in a tissue or a cell present in a whole animal) can
be used to associate PXR activity with a mammalian
30 disease state. For example, a compound that
activates PXR induces CYP3A. Thus, diseases in
which CYP3A activity is important are associated

with PXR, and compounds that activate or deactivate
PXR may be useful in prevention or treatment of such
diseases. By using the associating methods of this
invention, new PXR-associated diseases can be

5   discovered. Once these new associations are
discovered, new drugs for these diseases can be
identified by screening for compounds that activate
or deactivate PXR.

      An example of a compound suitable for use in

10   making disease associations in accordance with the
method described above is the compound of formula I:
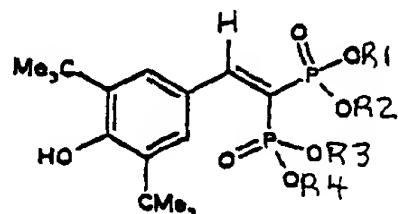
wherein each of R1, R2, R3 and R4 is, independently, C_1-C_6alkyl (linear or branched), preferably, C_2 or C_3alkyl (e.g., ethyl, n-propyl or iso-propyl), more preferably, C_2alkyl. The compounds

5   can be labelled with a detectable label, e.g., a radiolabel, e.g., tritium.

Another aspect of the invention relates to transgenic animals that express hPXR. For example, transgenic mice can be generated that express the

10   hPXR gene as well as the endogenous mouse PXR gene. Mice can also be generated in which the endogenous PXR gene is knocked out and then replaced by the hPXR gene. Transgenic aminals can be generated that express isoforms of hPXR as well as mutant alleles

15   of the gene. Transgenic animals developed by these methods can be used to screen compounds for drug interactions and toxicities, and to study the regulation of CYP3A in vivo.

A further aspect of the present invention

20   relates to diagnostic assays that can be used to screen for mutations in hPXR that alter the ability of the receptor to induce CYP3A4 gene expression. These assays can be based on the sequencing of the hPXR gene, on hybridization approaches designed to

25   detect sequence changes or polymorphisms, or the use of antibodies to distinguish wild-type from mutant/polymorphic hPXR. Changes that result in alteration of the DNA binding or ligand binding characteristics of hPXR can be expected to have a

30   significant impact on hPXR activity. A mutation or polymorphism in hPXR can be indicative of a patient at increased risk of suffering an adverse reaction

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to a drug as a result of unusual rates of drug metabolism.

The invention also relates to antibodies, polyclonal or monoclonal, that are specific for

5   hPXR, and antigen binding fragments thereof (e.g., Fab fragments). The antibodies can be generated in accordance with standard techniques using intact hPXR or a fragment thereof as defined above. The antibodies can be used, for example, in assays to

10   detect the presence of the receptor. Further, the antibodies can be used in hPXR purification protocols.

The invention also relates to kits suitable for use, for example, in one or more method described

15   above. The kits can include hPXR (or fragment thereof) or nucleic acid encoding same or antibodies as described above. The kit can also include compounds that bind hPXR, such as GW-485801. The hPXR, nucleic acid and/or antibody can be present in

20   the kit disposed within a container means. The kit can also include ancillary reagents and buffers, etc., to facilitate practice of the specific method.

Certain aspects of the present invention are described in greater detail in the non-limiting

25   Examples that follow.

                                EXAMPLES

The following experimental details are relevant to the specific Examples that follow.

Chemicals

Dexamethasone-t-butylacetate and RU486 were
purchased from Research Plus, Inc. (Bayonne, NJ) and
Biomol (Plymouth Meeting, PA), respectively. All
5   other compounds were purchased from either Sigma
Chemical Co. (St. Louis, MO) or Steraloids, Inc.
(Wilton, NH).

Molecular cloning of hPXR cDNAs

An EST was identified in the Incyte database
10   (clone identification number 2211526) that contained
nucleotides 444-2111 of the hPXR sequence. An
oligonucleotide derived from this EST sequence (5'
CTGCTGCGCATCCAGGACAT 3') (SEQ ID NO:1) was used to
screen a pCMV-SPORT human liver cDNA library
15   (Gibco/BRL) using Gene Trapper solution
hybridization cloning technology (Gibco/BRL). Two
clones were obtained that encoded hPXR, one
containing nucleotides 1-2125, the other containing
nucleotides 102-2118. The sequence of the longer is
20   shown in Figure 1A. Sequences were aligned and
analyzed by the University of Wisconsin Genetics
Computer Group programs.

Plasmids

The expression vector pSG5-hPXR was generated
25   by PCR amplification and subcloning of nucleotides
1-1608 of the hPXR clone into the pSG5 expression
vector (Strategene). pSG5-hPXR ATG was generated by
PCR amplification of cDNA encoding amino acids 1-434
of hPXR using oligonucleotides 5'-
30   GGGTGTGGGGAATCCACCACCATGGAGGTGAGACCCAAAGAAAGC-3'
(SEQ ID NO:2) (sense) and 5'-

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GGGTGTGGGGGATCCTCAGCTACCTGTGATGCCG-3' (SEQ ID NO:3)
(antisense) and insertion into EcoRI/BamHI-cut pSG5.
The bacterial expression vector pGEX-hPXR was
generated by PCR amplification of cDNA encoding

5   amino acids 108-434 and insertion into pGEX-2T
(Pharmacia). The reporter plasmid (DR3)$_4$-tk-CAT was
generated by insertion of four copies of a double-
stranded oligonucleotide containing the CYP3A1 DR3
PXRE (5'-GATCAGACAGTTCATGAAGTTCATCTAGATC-3') (SEQ ID

10   NO:4) into the BamHI site of pBLCAT2 (Luckow et al,
Nucl. Acids Res. 15:5490 (1987)). The reporter
plasmid (IR6)$_3$-tk-CAT was generated by insertion of
three copies of the CYP3A4 IR6 PXRE (5'-
GATCAATATGAACTCAAAGGAGGTCAGTG-3') (SEQ ID NO:5) into

15   the BamHI site of pBL2CAT. The pRSET-SRC1.14
expression plasmid has been previously described
(Kliewer, S.A., et al. Cell 92:73-82 (1998)). All
constructs were confirmed by sequence analysis.

Cotransfection assays

20   CV-1 cells were plated in 24-well plates in DME
medium supplemented with 10% charcoal-stripped fetal
calf serum at a density of 1.2 x 10^5 cells per well.
In general, transfection mixes contained 33 ng of
receptor expression vector, 100 ng of reporter

25   plasmid, 200 ng of β-galactosidase expression vector
(pCH110, Pharmacia), and 166 ng of carrier plasmid.
Cells were transfected overnight by lipofection
using Lipofectamine (Life Technologies, Inc.),
according to the manufacturer's instructions. The

30   medium was changed to DME medium supplemented with
10% delipidated calf serum (Sigma) and cells were

incubated for an additional 24 hours. Cell extracts
were prepared and assayed for CAT and β-
galactosidase activities as previously described
(Lehmann et al, J. Biol. Chem. 270:12953-12956
5   (1995)).

Northern analysis

An approximately 1.0 kb fragment encoding the
LBD of hPXR was [^{32}P]-labeled by random priming and
used to probe mouse multiple tissue Northern blots
10   (Clontech). Blots were hybridized in ExpressHyb
solution (Clontech) at 42°C overnight. Final washes
were performed with 0.1x SSC, 0.1% SDS at 58°C.

Band shift assays

hPXR, mPXR1, and hRXR were synthesized in
15   vitro using the TNT rabbit reticulocyte lysate
coupled in vitro transcription/translation system
(Promega) according to the manufacturer's
instructions. Gel mobility shift assays (20 µl)
contained 10 mM Tris (pH 8.0), 40 mM KCl, 0.05% NP-
20   40, 6% glycerol, 1 mM DTT, 0.2 µg of poly(dI-dC) and
2.5 µl each of in vitro synthesized PXR and RXR
proteins. Competitor oligonucleotides were included
at a 10-fold or 50-fold excess. After a 10 min
incubation on ice, 10 ng of [^{32}P]-labeled
25   oligonucleotide was added and the incubation
continued for an additional 10 min. DNA-protein
complexes were resolved on a 4% polyacrylamide gel
in 0.5X TBE (1X TBE = 90 mM Tris, 90 mM boric acid,
2 mM EDTA). Gels were dried and subjected to
30   autoradiography at -70°C. The following

oligonucleotides were used as either radiolabeled
probes or competitors (sense strand is shown):

CYP3A4 IR6:   5' GATCAATATGAACTCAAAGGAGGTCAGTG 3'
(SEQ ID NO:6)

5   CYP3A4 IR6m1 5' GATCAATATGTTCTCAAAGGAGAACAGTG 3'
(SEQ ID NO:7)

CYP3A4 IR6m2 5' GATCAATAACAACTCAAAGGAGGTCAGTG 3'
(SEQ ID NO:8)

CYP3A1 DR3:   5' GATGCAGACAGTTCATGAAGTTCATCTAGATC
10 3' (SEQ ID NO:9).

CARLA

GST-hPXR fusion protein was expressed in
BL21(DE3)plysS cells and bacterial extracts prepared
by one cycle of freeze-thaw of the cells in Protein
15 Lysis Buffer containing 10 mM Tris, pH 8.0, 50 mM
KCl, 10 mM DTT, and 1% NP-40 followed by
centrifugation at 40,000 x g for 30 minutes.
Glycerol was added to the resulting supernatant to a
final concentration of 10%. Lysates were stored at
20 -80°C. [^{35}S]SRC1.14 was generated using the TNT
rabbit reticulocyte system (Promega) in the presence
of Pro-Mix (Amersham). Coprecipitation reactions
included 25 µl of lysate containing GST-hPXR fusion
protein, 25 µl Incubation Buffer (50 mM KCl, 40 mM
25 HEPES pH 7.5, 5 mM ß-mercaptoethanol, 1% Tween-20,
1% non-fat dry milk), 5 µl [^{35}S]SRC1.14, and vehicle
(1% DMSO) or compounds as indicated. The mixtures
were incubated for 25 minutes at 4°C with gentle
mixing prior to the addition of 15 µl of
30 glutathione-sepharose 4B beads (Pharmacia) that had
been extensively washed with Protein Lysis Buffer.
Reactions were incubated with gentle mixing at 4°C

for an additional 25 min. The beads were pelleted
at 3000 rpm in a microfuge and washed 3 times with
Protein Incubation Buffer containing either vehicle
alone, dexamethasone-t-butylacetate, rifampicin, or
5   clotrimazole. After the last wash, the beads were
resuspended in 25 μl of 2X SDS-PAGE sample buffer
containing 50 mM DTT. Samples were heated at 100°C
for 5 minutes and loaded onto a 10% Bis-Tris PAGE
gel. Gels were dried and subjected to
10 autoradiography.

EXAMPLE 1

Molecular Cloning and
Tissue Expression Pattern of hPXR

A human EST was identified in the Incyte
15 LifeSeq® proprietary database that was highly
homologous to a region of mPXR1 (Kliewer et al, Cell
92:73-82 (1998)). Two larger clones were isolated
in a screen of a human liver cDNA library using an
oligonucleotide within the EST as a probe. The
20 longest of these clones was 2146 bp in length
(Fig. 1A) and encoded a new member of the nuclear
receptor superfamily that was 97% and 76% identical
to mPXR1 in the DNA binding domain (DBD) and ligand
binding domain (LBD), respectively (Fig. 1B). In
25 terms of other members of the nuclear receptor
superfamily, hPXR was most closely related to the
Xenopus laevis orphan receptor ONR1 (Smith et al,
Nucl. Acids Res. 22:66-71 (1994)) and the vitamin D
receptor (Fig. 1B). Notably, the hPXR sequence
30 lacked an AUG initiator codon in between an in-frame
stop codon (nucleotides 205-207 in the hPXR

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sequence) and the start of the region encoding the
DBD. However, transfection experiments performed in
CV-1 cells with the hPXR clone and a reporter
plasmid containing four copies of an established
5   mPXR binding site from the rat CYP3A1 gene promoter
inserted upstream of the minimal thymidine kinase
(tk) promoter and the chloramphenicol
acetyltransferase (CAT) gene (Kliewer et al, Cell
92:73-82 (1998)) demonstrated that the hPXR clone
10   encoded a functional nuclear receptor that was
activated efficiently by dexamethasone-t-
butylacetate, a known mPXR1 ligand (Kliewer et al,
Cell 92:73-82 (1998)) (Fig. 1C).

Examination of the hPXR sequence revealed an
15   in-frame CUG codon (nucleotides 304-306) surrounded
by a favorable Kozak sequence (Kozak, J. Biol. Chem.
266:19867-19870 (1991)). There is precedent for the
use of CUG codons to initiate translation of
eukaryotic proteins, including the nuclear receptor
20   RARβ4 (Kozak, J. Biol. Chem. 266:19867-19870 (1991),
Nagpal et al, Proc. Natl. Acad. Sci. USA 89:2718-
2722 (1992)). Initiation of translation at this CUG
codon would yield a protein of 434 amino acids,
three longer than mPXR1, with a predicted MW of 49.7
25   kD. In order to determine whether translation of
the hPXR cDNA initiated at the CUG codon, hPXR RNA
containing the wild-type 5' region was translated in
the presence of [^{35}S]methionine using rabbit
reticulocyte lysates. As a control, hPXR RNA, in
30   which this CUG codon had been mutated to the optimal
AUG (hPXR AUG), was also translated *in vitro*.
Translation of the wild-type hPXR RNA resulted in an

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approximately 50 kD protein that co-migrated with
the translation product of hPXR AUG RNA (Fig. 1D,
open arrow with asterisk). This 50 kD product was
not produced when hPXR antisense RNA was used in the
5   translation reaction. Much lower amounts of an
approximately 53 kD translation product were also
produced in translation reactions performed with
hPXR RNA (Fig. 1D, bent arrow), indicating that a
small amount of translation initiated at other non-
10   AUG codons upstream of the CUG codon. However, the
results indicate that the CUG codon represents the
principal translation initiation site for hPXR
containing a functional DBD.

The tissue expression pattern of hPXR was next
15   examined via Northern analysis using blots
containing poly(A)+ RNA prepared from multiple adult
tissues. hPXR mRNA was expressed most abundantly in
liver and was also present in the colon and small
intestine (Fig. 2). Three transcripts of different
20   size were detected in each of these tissues: a
prominent 2.6 kb product and two less abundant
messages of approximately 4.3 kb and 5 kb. It was
recently shown that the mPXR gene is also abundantly
expressed in liver and small intestine (Kliewer et
25   al, Cell 92:73-82 (1998)). Whereas mPXR message was
also detected at low levels in stomach and kidney,
mRNA for hPXR was not detected in these tissues
(Fig. 2). Thus, both hPXR and mPXR are most
abundantly expressed in the liver and tissues of the
30   gastrointestinal tract; however, there are
differences in PXR expression patterns in mice and
humans.

EXAMPLE 2

hPXR Activates Transcription Through
a Response Element in the CYP3A4 Gene Promoter

Several lines of evidence have been provided
that mPXR1 regulates CYP3A1 gene expression: mPXR1
was activated by compounds known to activate CYP3A1
gene expression including glucocorticoids and
antiglucocorticoids, mPXR1 and CYP3A1 gene
expression colocalized in the liver and small
intestine, and mPXR1 bound to a response element in
the CYP3A1 gene promoter that had previously been
determined to confer responsiveness to
glucocorticoids and antiglucocorticoids (Kliewer et
al, Cell 92:73-82 (1998), Quattrochi et al, J. Biol.
Chem. 270:28917-28923 (1995), Huss et al, J. Biol.
Chem. 93:4666-4670 (1996)). The findings that the
CYP3A4 gene is also expressed in the liver and
intestine and that this expression is induced in
response to glucocorticoids and antiglucocorticoids
(Molawa et al, Proc. Natl. Acad. Sci. USA 83:5311-
5315 (1986), Kocarek et al, Drug Met. Dispos.
23:415-421 (1995)) led to the investigation of
whether hPXR regulates CYP3A4 gene expression.

The induction of CYP3A4 expression in response
to dexamethasone and rifampicin has been localized
to an approximately 20 bp region of the promoter
that contains two copies of the nuclear receptor
half-site sequence AG(G/T)TCA organized as an
inverted repeat (IR) and separated by 6 base pairs,
an IR6 motif (Barwick et al, Molec. Pharmacol.
50:10-16 (1996)) (Fig. 3B). This IR6 motif is
highly conserved in the promoters of CYP3A gene

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family members of several species (Barwick et al,
Molec. Pharmacol. 50:10-16 (1996)). Interestingly,
this half-site configuration is very different from
that found in the CYP3A1 PXR response element (PXRE)

5   which contains two half-sites organized as a direct
repeat (DR) with a 3 nucleotide spacer, a DR3 motif
(Kliewer et al, Cell 92:73-82 (1998)). To determine
whether hPXR could regulate transcription through
the IR6 motif, a reporter plasmid was generated

10   containing three copies of the CYP3A4 IR6 response
element upstream of the tk promoter and CAT gene.
Cotransfection assays were performed with the (IR6)$_3$-
tk-CAT reporter and pSG5-hPXR ATG expression
plasmids in CV-1 cells that were either treated with

15   vehicle alone or 10 µM dexamethasone-t-butylacetate.
hPXR induced reporter levels in the presence of
dexamethasone-t-butylacetate (Fig. 3A),
demonstrating that hPXR can activate transcription
through the CYP3A4 IR6 motif.

20   In order to determine whether hPXR interacted
directly with the CYP3A4 IR6 response element, band
shift assays were performed. Since mPXR1 binds to
DNA as a heterodimer with RXR (Kliewer et al, Cell
92:73-82 (1998)), it was suspected that hPXR would

25   require RXR for high-affinity interactions with DNA.
Neither hPXR nor RXR bound to a radiolabeled
oligonucleotide containing the CYP3A4 IR6 motif on
their own (Fig. 3C). However, hPXR and RXR bound
efficiently as a heterodimer to the IR6 PXRE. The

30   hPXR/RXR complex was competed efficiently by
unlabeled oligonucleotides encoding either the IR6
PXRE from the CYP3A4 promoter or the DR3 PXRE from

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the CYP3A1 promoter that it was previously defined
as a mPXR1/RXR binding site (Kliewer et al, Cell
92:73-82 (1998)) (Fig. 3C). Thus, the hPXR/RXR
heterodimer interacted efficiently with two response
5 elements with remarkably different architecture.
Little or no competition was seen when competitor
oligonucleotides were used that contained mutations
in either the 5' half-site or both half-site
sequences of the IR6 PXRE (Fig. 3C). The same
10 binding profile was observed when the mPXR1 was
substituted for hPXR (Fig. 3C). It was concluded
from these experiments that hPXR binds efficiently
to the CYP3A4 IR6 PXRE as a heterodimer with RXR,
and that hPXR and mPXR1 have very similar DNA
15 binding profiles.

EXAMPLE 3

Differential Activation of Human and mPXR

CYP3A4 gene expression is induced in response
to a remarkable array of xenobiotics, including
20 synthetic steroids (Kocarek et al, Drug Met. Dispos.
23:415-421 (1995), Schuetz et al, J. Biol. Chem.
259:2007-2012 (1984), Heuman et al, Mol. Pharmacol.
21:753-760 (1982), Schulte-Hermann et al, Cancer
Res. 48:2462-2468 (1988)), macrolide antibiotics
25 (Wrighton et al, Biochem. 24:2171-2178 (1985)),
antimycotics (Hostetler et al, Mol. Pharmacol.
35:279-285 (1989)), HMG-CoA reductase inhibitors
(statins) (Kocarek et al, Toxicol. Appl. Pharmacol.
120:298-307 (1993), Schuetz et al,. Hepatology
30 18:1254-1262 (1993)), and phenobarbital-like

compounds (Heuman et al, Mol. Pharmacol. 21:753-760
(1982)). It was next determined whether hPXR might
mediate the effects of some or all of these
compounds on CYP3A4 expression. CV-1 cells were
5 cotransfected with the pSG5-hPXR ATG expression
plasmid and the $(IR6)_3$-tk-CAT reporter plasmid, and
the cells were treated with micromolar
concentrations of a number of compounds that are
known to induce CYP3A gene expression in humans
10 and/or rodents. As shown in Fig. 4A, hPXR was
activated by the synthetic steroids dexamethasone,
dexamethasone-t-butylacetate, PCN, RU486,
spironolactone, and cyproterone-acetate.
Dexamethasone-t-butylacetate and RU486 were the most
15 efficacious activators of hPXR among the synthetic
steroids tested. Notably, the antibiotic rifampicin
and the antimycotic clotrimazole were both
efficacious activators of hPXR (Fig. 4A). The
antihypercholesterolemic drug lovastatin also
20 activated hPXR as did phenobarbital and the
organochlorine pesticide transnonachlor (Fig. 4A).
Thus, hPXR is activated by a remarkably diverse
group of synthetic compounds that are known to
induce CYP3A4 gene expression (Fig. 4B).
25 Several naturally-occurring C21 steroids were
also tested on hPXR that were previously shown to
activate mPXR1 (Kliewer et al, Cell 92:73-82
(1998)). Pregnenolone, progesterone, and 5 β-
pregnane-3,20-dione all activated hPXR roughly 4-
30 fold. The 17-hydroxy derivatives of pregnenolone
and progesterone were weak activators of hPXR (Fig.
4A). These natural steroids all activated hPXR in

transient transfection assays with EC_{50} values >10 µM, suggesting that they are unlikely to be natural hPXR ligands. However, related pregnanes or pregnane metabolites may serve as natural hPXR

5   ligands.

Analyses of the effects of chemical inducers of CYP3A gene expression in primary hepatocytes obtained from either rodents or humans have revealed significant interspecies differences (Barwick et al,

10   Molec. Pharmacol. 50:10-16 (1996), Kocarek et al, Drug Met. Dispos. 23:415-421 (1995)). For example, rifampicin is an efficacious inducer of CYP3A4 gene expression in human hepatocytes but has little or no effect on CYP3A1 levels in rat hepatocytes. In

15   contrast, PCN has marked effects on CYP3A levels in rat hepatocytes but only modest effects in human hepatocytes. To examine whether differences in PXR activation profiles might account for these interspecies variations, the same panel of compounds

20   was tested on mPXR1. As shown in Fig. 4A, there were marked differences in the response profiles of the mouse and human homologs of PXR. Whereas rifampicin was an efficacious activator of hPXR, it was only a weak activator of mPXR1 (Fig. 4A).

25   Clotrimazole, lovastatin and phenobarbital were also more efficacious activators of hPXR than mPXR1. In contrast, PCN only activated hPXR approximately 3-fold but activated mPXR1 roughly 9-fold (Fig. 4A). Taken together, these data indicate that much of the

30   interspecies variability in CYP3A regulation may be due to differences in PXR activation profiles.

The panel of chemicals that induce CYP3A expression was also profiled on the human glucocorticoid receptor (GR). As shown in Fig. 4A, only dexamethasone and dexamethasone-t-butylacetate

5   were efficacious activators of the GR. None of the other compounds activated the GR >1.5-fold (Fig. 4A). In contrast to a recent report (Calleja et al, Nature Med. 4:92-96 (1998)), activation of the GR by rifampicin was not observed. Since this previous

10   work was performed in HepG2 cells, it may be that rifampicin is differentially metabolized in various cell lines. As expected, neither pregnenolone, progesterone, nor their 17-hydroxy derivatives had an effect on GR activity (Fig. 4A). Thus, the broad

15   activation profile that was observed for the human and mouse homologs of PXR with inducers of CYP3A gene expression is not a general property of other steroid hormone receptors.

       In the absence of high-affinity radioligands,

20   coactivator-based assays have been used as a biochemical means to determine whether compounds that activate orphan nuclear receptors do so through direct interactions with the protein (Kliewer et al, Cell 92:73-82 (1998), Krey et al, Mol. Endocrinol.

25   11:779-791 (1997)). These assays are predicated on the finding that ligands induce the interaction of nuclear receptors with accessory proteins, termed coactivators (Krey et al, Mol. Endocrinol. 11:779-791 (1997)). It was recently demonstrated that

30   several steroidal activators of mPXR1, including dexamethasone-t-butylacetate and PCN, promote the interaction of the mPXR1 LBD with a 14 kD fragment

of the steroid receptor coactivator 1 (SRC1.14)
(Kliewer et al, Cell 92:73-82 (1998)). In order to
examine whether the structurally-diverse compounds
that activate hPXR do so by acting as ligands, three
5   of the more potent activators representing different
chemical classes were selected, dexamethasone-t-
butylacetate, rifampicin, and clotrimazole, for
testing in the coactivator-receptor ligand assay
(CARLA). The LBDs of hPXR and mPXR1 were expressed
10   in *E. coli* as fusion proteins with glutathione-S-
transferase (GST), and SRC1.14 was synthesized *in
vitro* in the presence of [^{35}S]methionine and
[^{35}S]cysteine. As shown in Fig. 4C, dexamethasone-t-
butylacetate, rifampicin and clotrimazole each
15   promoted the interaction of [^{35}S]SRC1.14 with GST-
hPXR. Consistent with the results of the
transfection studies, dexamethasone-t-butylacetate
induced an efficient interaction between GST-mPXR1
and [^{35}S]SRC1.14 whereas rifampicin and clotrimazole
20   did not (Fig. 4C). Taken together, these data
indicate that structurally-divergent compounds can
serve as hPXR ligands, and that the human and mouse
homologs of PXR differ significantly in terms of
their ligand binding properties.

25   EXAMPLE 4

                        Transfection Assay

      Plasmids: GAL4-hPXR chimera and UAS-tk-SPAP
reporters. The GAL4-hPXR expression constructs
contain the translation initiation sequence and
30   amino acids 1 to 147 of the yeast *S. crevisiae*
transcription factor GAL4 in the pSG5 expression

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vector (Statagene). Amino acids 108 to 434 of hPXR
are amplified by polymerase chain reaction (PCR)
using vent polymerase (New England Biolads) and
inserted C-terminal to the GAL4 sequences. The UAS-
5   tk-SPAP reporter contains 5 copies of the GAL4
binding site upstream of the tk promoter and the CAT
gene (Berger et al, Gene 66:1 (1988)).

Transfection assay: SPAP reporter. CV-1 cells
are plated in DME medium supplemented with 10%
10   delipidated fetal calf serum at a density of 2.4×10^4
cells per well in a 96-well plate (Costar) 16-24 h
before transfection. In general, 8.0 ng of reporter
plasmid, 25.0 ng of β-galactosidase expression
vector (pCH110, Pharmacia), and 2.0 ng of GAL4-hPXR
15   expression vector are mixed with carrier DNA
(pBluescript, Stratagene) to a total of 80 ng of DNA
per well in a volume of 10ml optiMEM I medium (Life
Technologies). To this, a second mix, containing
9.3 ml optiMEM I medium and 0.7 ml of
20   LIPOFECTAMINE^{TM} (Life Technologies), is added.
After 30 min., an additional 80ml of optiMEM I
medium are added and the combined mix is then
applied to the cells. Sixteen hours later, the
medium is changed to DME medium supplemented with
25   10% delipidated and heat inactivated fetal calf
serum and the test compound at a concentration of
10^{-5}M. After incubation for 24 h, SPAP activity and
β-galactosidase activity are measured by directly
adding to the medium 200ml substrate mix (16mM
30   o-nitrophenyl β-D-galactopyranoside (Sigma), 120mM
fluorescein diphosphate (Molecular Probes), 0.16%
Triton X-100, 160mM diethanolamine pH9, 44.8mM NaCl,

and 0.8mM MgCl$_2$). Alternatively, alkaline

phosphatase and β-galactosidase activities are

measured separately using standard protocols.

Briefly, cells are lysed by adding 25ml 0.5% Triton

5   X-100 to the supernatant. To 40ml cell lysate,

200ml β-galactosidase substrate reagent (36mM

o-nitrophenyl β-D-galactopyranoside, 1.25mM MgCl$_2$,

2.8mM NaCl, 4.4M β-mercaptoethanol) or 200ml

alkaline phosphatase substrate reagent (2.5mM

10   p-nitrophenyl phosphate, 0.5mM MgCl$_2$, 20mM NaCl, 1 M

diethanolamine pH 9.85) are added and incubated for

1 h. Alkaline phosphatase activity is expressed as

fold activation relative to that observed with

vehicle alone (normalized to β-galactosidase

15   activity which serves as internal control standard

for transfection efficiency).

EXAMPLE 5

Synthesis of [^{3}H]GW-485801

(i)   The Preparation of [^{3}H]3,5-Ditertbutyl-4-hydroxy

20   benzaldehyde.

3,5-Diterbutyl-4-hydroxy benzaldehyde, 5 mg

(20.6 μmol) and Crabtree catalyst, 7.5 mg

(9.3 μmol), were dissolved in 2 ml dichloromethane

and stirred under 10 Ci tritium gas for 5 hours.

25   The solution was then evaporated to dryness, and

labile tritium was removed by repeated evaporations

from methanol. The residue was redissolved in

methanol, 10 ml, counted and analyzed.

Yield = 800 mCi.

Radiochemical purity by TLC on silica in
hexane:ethyl acetate (80:20) was approximately 50%.

The crude material was evaporated to 1 ml and
purified by preparative plate chromatography on a
5   single 500 µm silica plate, eluting in hexane:ethyl
acetate (85:15). The plates were viewed under UV,
the band corresponding to required aldehyde was
collected and the product extracted into ethyl
acetate. This was evaporated to dryness and
10   redissolved in dichloromethane, counted and
analyzed.

Yield = 370 mCi.

TLC as above showed a singly labelled, specific
activity 23 Ci/mmol.

15   (ii) The Preparation of [³H]GW-485801

The product from (i) above (370 mCi at 23
Ci/mmol, 16 µmol) was evaporated to dryness,
redissolved in THF, 1 ml, and cooled in an ice bath
with stirring. 1M Titanium (IV) chloride in
20   toluene, 55 µl, 55 µmol, was added, immediate yellow
color formed. Tetraethyl methylenediphosphonate, 75
µl, of a THF solution at 110 mg/ml, 28.6 µmol, was
added, followed by N-methyl morpholine, 8.1 µl,
7.5 mg, 74 µmol. This caused a deep blue color.
25   The solution was then stirred at room temperature
for 4 hours.

TLC analysis on silica in ethyl
acetate:methanol (90:10) showed approximately 60% of
the radioactivity to correspond to inactive
30   GW-485801.

(iii) The Purification of [³H]GW-485801

The crude product was purified by preparative plate chromatography on 2 x 1mm silica plates, eluting in ethyl acetate:methanol (90:10). The
5   plates were viewed under UV, the band corresponding to required product was collected and the product extracted into ethyl acetate:methanol (90:10). This was evaporated to dryness and redissolved in nitrogen-flushed ethanol, 30 ml. This was a yellow
10 solution.

Yield = 180 mCi.

(iv) The Analysis of [³H]GW-485801

The purified product resulting from (iii) was analyzed by TLC, HPLC, mass spectroscopy and T-NMR.
15   TLC showed a radiochemical purity of 99%.

HPLC showed a radiochemical purity of 98.9%.

In both of the above systems, the radioactive peak co-eluted with inactive GW-485801.

Mass spectroscopy showed a specific activity of
20 23 Ci/mmol, the isotope distribution being 18.4% unlabelled, 81.6% 1 x ³H. The spectrum of the radioactive material was consistent with that of the inactive GW-485801.

T-NMR showed a single labelling position (peak
25 split into four signals by coupling to the phosphorus atoms) corresponding to labelling in the vinylic position of GW-485801. This corresponds to labelling in the aldehyde-H in the precursor.

A portion of the material was diluted to
30 1 mCi/ml with nitrogen-flushed ethanol and dispensed as 1 X 2 mCi pack. The remainder was stored at ~20°C (approximately 170 mCi).

EXAMPLE 6

Biotin-His6-PXR/RXRa Protein

The coding sequence representing amino acids
130-434 of human PXR (Genbank AF061056) was
5   subcloned into the pRSETa expression vector
(Invitrogen). Sequence encoding a polyhistidine tag
derived from an N-terminal PCR primer (MKKGHHHHHHG)
(SEQ ID NO:10) was fused in-frame. The resulting
encoded His6-PXR sequence was as follows:
10

MKKGHHHHHHGSERTGTQPLGVQGLTEEQRMMIRELMDAQMKTFDTTFSHFK
NFRLPGVLSSGCELPESLQAPSREEAAKWSQVRKDLCSLKVSLQLRGEDGSV
WNYKPPADSGGKEIFSLLPHMADMSTYMFKGIISFAKVISYFRDLPIEDQIS
LLKGAAFELCQLRFNTVFNAETGTWECGRLSYCLEDTAGGFQQLLLEPMLKF
15 HYMLKKLQLHEEEYVLMQAISLFSPDRPGVLQHRVVDQLQEQFAITLKSYIE
CNRPQPAHRFLFLKIMAMLTELRSINAQHTQRLLRIQDIHPFATPLMQELFG
ITGS (SEQ ID NO:11).

Restriction enzymes Nde I and Hind III were
20 used to release the cDNA fragment encoding amino
acids 225-462 of RXRα from BB5508 (pRSETa). The
fragment was ligated into the like-cut pET24a
expression plasmid (Novagen). The Bgl II, Hind III
fragment (contains T7 promoter, lac operator, RBS
25 and RXRa) of this construct was then cloned into the
BamH I, Hind III sites (removes tetracycline
resistance) of pACYC184 (BB5114). This allows for
expression of RXRα from the T7 promoter when grown
in BL21(DE3) cells and induced with IPTG. The
30 resulting encoded RXRα sequence was as follows:

-37-

MKKGSANEDMPVERILEAELAVEPKTETYVEANMGLNPSSPNDPVTNICQAA

DKQLFTLVEWAKRIPHFSELPLDDQVILLRAGWNELLIASFSHRSIAVKDGI

LLATGLHVHRNSAHSAGVGAIFDRVLTELVSKMRDMQMDKTELGCLRAIVLF

5   NPDSKGLSNPAEVEALREKVYASLEAYCKHKYPEQPGRFAKLLLRLPALRSI

GLKCLEHLFFFKLIGDTPIDTFLMEMLEAPHQMT (SEQ ID NO:12).

        The His6-PXR/pRSETa and RXRα/pACYC184 plasmids
    were cotransformed into the BL21(DE3) *E. coli*
10 strain. One-liter shake flask liquid cultures
    containing standard Luria-Bertani (LB) broth with
    0.05 mg/ml Ampicillin and 0.05 mg/ml Chloramphenicol
    were inoculated and grown at 22°C for 24 hours. The
    cells were induced with 0.05 mM IPTG for 4-6 hours
15 at 22°C then the cells were harvested by
    centrifugation (20 minutes, 3500 g, 4°C). The cell
    pellet was stored at -80°C. The cell pellet was
    resuspended in 250 ml Buffer A (50 mM Tris-Cl pH8.0,
    250 mM NaCl, 50 mM imidazole pH7.5). Cells were
20 sonicated for 3-5 minutes on ice and the cell debris
    was removed by centrifugation (45 minutes, 20,000g,
    4°C). The cleared supernatant was filtered through a
    0.45 mM filter and loaded on to a 50 ml ProBond [Ni⁺⁺
    charged] chelation resin (Invitrogen). After
25 washing to baseline with Buffer A, the column was
    washed with Buffer A containing 125 mM imidazle pH
    7.5. The His6-PXR/RXRα complex was eluted from the
    column using Buffer A with 300 mM imidazole pH 7.5.
    Column fractions were pooled and concentrated using
30 Centri-prep 30K (Amicon) units. The protein was
    subjected to size exclusion, using a column (26 mm X
    90 cm) packed with Sepharose S-75 resin (Pharmacia)

pre-equilibrated with 20mM Tris-Cl pH 8.0, 200 mM

NaCl, 5 mM DTT, 2.5 mM EDTA, pH 8.0. Column

fractions were pooled and concentrated as before.

The purified His6-PXR/RXRα was buffer exchanged by

5   gel filtration into PBS, resulting in an average

total molar protein concentration of 45mM. A five-

fold total molar excess of NHS-LC-Biotin (Pierce)

was added to this protein mixture in a minimal

volume of PBS. This solution was incubated with

10   gentle mixing for 60 minutes at ambient temperature,

approximately 23°C. The biotinylation modification

reaction was stopped by the addition of a 2000x

molar excess of Tris-HCl, pH 8. The biotin-His6-

PXR/RXRα was dialyzed at 4°C against 3 buffer

15   changes, each of at least 50 volumes, TBS pH 8

containing 5mM DTT, 2mM EDTA and 2% sucrose. The

biotin-His6-PXR/RXRα was subjected to mass

spectrometric analysis to reveal the extent of

modification by the reagent. The biotinylated

20   protein solution was frozen and stored at -80°C.

EXAMPLE 7

PXR Scintillation Proximity Assay (SPA)

Streptavidin-PVT SPA beads (AmershamPharmacia

cat # RPNQ0007) were resuspended in assay buffer (50

25   mM Tris HCl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mg/ml

essentially fatty acid free bovine serum albumin) at

0.5 mg/ml. Biotin-His6-PXR/RXRα was added to the

beads to a final concentration of 50 nM. The

receptors were allowed to couple to the SPA beads

30   for thirty minutes at room temperature. The

uncoupled receptor was removed by centrifuging the
SPA beads at 3000rpm for 5 minutes in a swinging
bucket rotor of a Rupp & Bowman Silencer centrifuge.
The receptor coated SPA beads were then resuspended

5   in assay buffer to 3.3 mg/ml. 100 µg (30 µL) of
receptor coated SPA beads were added to each well of
a 96-well Optiplate (Packard cat # 6005190). Each
well also contained [^{3}H]GW-485801 at final
concentrations ranging from 0.5 nM to 800 nM. Non-

10 specific binding was determined by addition of 10 µM
clotrimazole. The total volume in each well was 100
µL. The plates were sealed with TopSealA (Packard
cat # 6005185) and agitated momentarily to ensure
complete mixing. The plates were then allowed to

15 incubate at room temperature until equilibrium was
obtained. The plates were then counted on a
TopCount liquid scintillation counter (Packard)
using a protocol optimized for ^{3}H PVT SPA.
Triplicate samples in the absence (T samples) or

20 presence (NS samples) of clotrimazole were averaged
and specific binding was calculated using the
equation:

$$specific\ binding = T-NS$$

25

Plots of specific binding vs concentration of [^{3}H]GW-
485801 were generated (Fig. 6). Kd values were
determined using non-linear regression when the data
were fit to the equation of a rectangular hyperbola.

30   Test compounds were dissolved in DMSO at 10 mM
and diluted 1:10 in DMSO before serially diluting in
assay buffer. Compounds were typically tested at

concentrations ranging from 100µM to 0.3nM.
Streptavidin-PVT SPA beads (AmershamPharmacia cat #
RPNQ0007) were resuspended in assay buffer (50 mM
Tris HCl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mg/ml

5   essentially fatty acid free bovine serum albumin) at
0.5 mg/ml. Biotin-His6-PXR/RXRα was added to the
beads to a final concentration of 50 nM. The
receptors were allowed to couple to the SPA beads
for thirty minutes at room temperature. The

10   uncoupled receptor was removed by centrifuging the
SPA beads at 3000rpm for 5 minutes in a swinging
bucket rotor of a Rupp & Bowman Silencer centrifuge.
The receptor coated SPA beads were then resuspended
in assay buffer to 3.3 mg/ml. 100 µg (30 µL) of

15   receptor coated SPA beads was added to each well of
a 96-well Optiplate (Packard cat # 6005190). Each
well also contained [^{3}H]GW-485801 at a final
concentration of 25 nM and test compound or an equal
volume of assay buffer. Non-specific binding was

20   determined by addition of 10 µM clotrimazole. The
total volume in each well was 100 µL. The plates
were sealed with TopSealA (Packard cat # 6005185)
and agitated momentarily to ensure complete mixing.
The plates were then allowed to incubate at room

25   temperature until equilibrium was obtained,
approximately 1.5 hours. The plates were then
counted on a TopCount liquid scintillation counter
(Packard) using a protocol optimized for ^{3}H PVT SPA
and programmed to correct for color quenching.

30   Values for "% [^{3}H]GW-485801 Bound" were calculated
using the following equation:

$$\% \ [^{3}\text{H}]\text{GW-485801 Bound} = 100*[(C_{DPM} - NS_{DPM})/(T_{DPM} - NS_{DPM})]$$

where C_{DPM} is the DPM value from a well containing a
test compound, NS_{DPM} is the average of the DPM values
from the "non-specific" wells which contained 10 µM
clotrimazole, T_{DPM} is the average of the DPM values
from the "total" wells which contained no added
compounds. Graphs of $\%[^{3}\text{H}]$GW-485801 Bound vs
concentration were generated for each test compound
and IC50 values were determined using non-linear
regression (see Table 1).

Table 1

Compound	IC50 (µM)
GW-485801	0.58
Clotrimazole	1.3
Rifampicin	2.4
5b-pregnane-3,20-dione	1.0

*   *   *

All documents cited above are hereby
incorporated in their entirety by reference.

One skilled in the art will appreciate from a
reading of this disclosure that various changes in
form and detail can be made without departing from
the true scope of the invention.

WHAT IS CLAIMED IS:

1.   An isolated human nuclear receptor
that binds to a cytochrome P-450 monooxygenase
promoter, or a DNA binding or ligand binding
domain thereof.

2.   The receptor according to claim 1
wherein the promoter is a cytochrome P-450
monooxygenase 3A4 (CYP3A4) promoter.

3.   The receptor according to claim 2
wherein said receptor is hPXR.

4.   An isolated human nuclear receptor
having the amino acid sequence given Figure 1,
or a fragment thereof, of at least
30 consecutive amino acids.

5.   A fusion protein comprising a DNA
binding or ligand binding domain of hPXR and a
non-hPXR-derived sequence.

6.   An isolated nucleic acid comprising a
sequence encoding the receptor of claim 1 or 4
or the fusion protein of claim 5.

7.   A construct comprising the nucleic
acid of claim 6 and a vector.

8.   A host cell comprising the construct
of claim 7.

9.   A method of making the receptor of
claim 3, or fragment thereof, comprising:

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culturing a host cell containing an expression construct comprising a sequence encoding said receptor, or fragment thereof, operably linked to a promoter, under conditions such that said receptor, or fragment thereof, is produced, and

isolating said receptor, or fragment thereof.

10.   A method of screening a test compound for its ability to induce CYP3A4 gene expression comprising

i)   contacting said test compound with the ligand binding domain of hPXR,

ii)   determining whether said test compound binds to said ligand binding domain,

wherein binding of the test compound to said ligand binding domain is indicative of a compound that induces CYP3A4 gene expression.

11.   A method of screening a test compound for its ability to activate or inhibit hPXR comprising:

i)   preparing an expression vector comprising a sequence encoding a DNA binding domain and a hPXR ligand binding domain;

ii)   preparing a reporter construct comprising a DNA binding site recognized by said DNA binding domain operably linked to a reporter gene,

iii)   introducing said expression vector and said reporter construct into compatible host cells,

-44-

iv)   incubating said cells resulting from
step (iii) with said test compound, and

v)   determining the level of expression
of said reporter gene,

wherein enhancement of expression of
said reporter gene in the presence of said test
compound indicates that said test compound can
activate hPXR, and

wherein inhibition of expression of
said reporter gene in the presence of said test
compound indicates that said test compound can
inhibit hPXR.

12.   A compound that induces CYP3A4
identified by the method of claim 10.

13.   A compound that activates hPXR
identified by the method of claim 11.

14.   A method of modulating function of a
cell mediated by PXR comprising contacting said
cell with a compound identified using the
method of claim 11 that activates PXR under
conditions such that said activation is
effected and said function is thereby
modulated.

15.   A method of modulating function of a
cell mediated by PXR comprising contacting said
cell with a compound identified using the
method of claim 11 that inhibits PXR under
conditions such that said inhibition is

effected and said function is thereby
modulated.

16. The method according to claim 14 or
15 wherein said compound is of formula I:
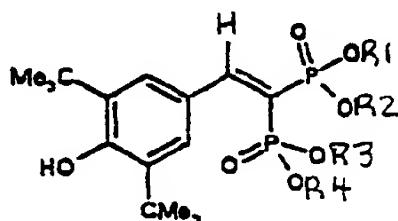
wherein R1, R2, R3 and R4 are, independently,
C_1-C_6alkyl, linear or branched.

17. The method according to claim 14 or
15 wherein said cell is a cultured cell.

18. The method according to claim 14 or
15 wherein said cell is present in a tissue.

19. The method according to claim 14 or
15 wherein said cell is present in an animal.

20. A method for associating a particular
disease or condition with modulation of PXR
comprising
    contacting a compound that binds to PXR
specifically with PXR present in a cell under
conditions such that said binding is effected

and a functional activity of said cell mediated
by PXR is thereby modulated,

detecting said modulation of said
functional activity and associating said
modulation of said functional activity with a
disease or condition and thereby associating
said disease or condition with modulation of
PXR.

21.   The method according to claim 20
wherein said compound is of formula I:
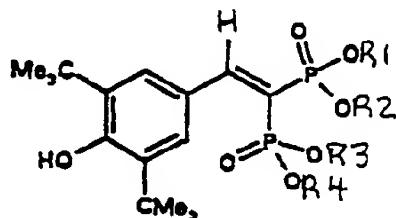
wherein R1, R2, R3 and R4 are,
independently, C_1-C_6alkyl, linear or branched.

22.   The method according to claim 21
wherein said compound is GW-485801.

23.   A method of preventing or treating a
disease or condition that has been associated
with modulation PXR by the method of claim 20,
comprising administering to a patient in need
thereof a therapeutically effective amount of
an agent that modulates the activity of PXR so
that said prevention or treatment is effected.

24 .　The method according to claim 23
wherein said agent is GW-485801.

```
1     TGAAATATAGGTGAGAGACAAGATTGTCTCATATCCGGGGAAATCATAACCTATGACTAG
61    GACGGGAGGAGGAAGCACTGCCTTTACTTCAGTGGGAATCTCGGCCTCAGCCTGCAAGCC
121   AAGTGTTCACAGTGAGAAAAGCAAGAGAATAAGCTAATACTCCTGTCCTGAACAAGGCAG
181   CGGCTCCTTGGTAAAGCTACTCCTTGATCGATCCTTTGCACCGGATTGTTCAAAGTGGAC
241   CCCAGGGGAGAAGTCGGAGCAAAGAACTTACCACCAAGCAGTCCAAGAGGCCCAGAAGCA
301   AACCTGGAGGTGAGACCCAAAGAAAGTCTGAACCATGTCGACTTTGTACACTGTGAGGAC
          L  E  V  R  P  K  E  S  W  N  H  A  D  F  V  H  C  E  D   19
361   ACAGAGTCTGTTCCTGGAAAGCCCAGTGTCAACGCAGATGAGGAAGTCGGAGGTCCCCAA
          T  E  S  V  P  G  K  P  S  V  N  A  D  E  E  V  G  G  P  Q   39
421   ATCTGCCGTGTATGTGGGGACAAGGCCACTGGCTATCACTTCAATGTCATGACATGTGAA
          I  C  R  V  C  G  D  K  A  T  G  Y  H  F  N  V  M  T  C  E   59
481   GGATGCAAGGGCTTTTTCAGGAGGGCCATGAAACGCAACGCCCGGCTGAGGTGCCCCTTC
          G  C  K  G  F  F  R  R  A  M  K  R  N  A  R  L  R  C  P  F   79
541   CGGAAGGGCGCCTGCGAGATCACCCGGAAGACCCGGCAGTGCCAGGCCTGCCGCCTG
          R  K  G  A  C  E  I  T  R  K  T  R  Q  C  Q  A  C  R  L   99
601   CGCAAGTGCCTGGAGAGCGGCATGAAGAAGGAGATGATCATGTCCGACGAGGCCGTGGAG
          R  K  C  L  E  S  G  M  K  K  E  M  I  M  S  D  E  A  V  E   119
661   GAGAGGCGGGCCCTTGATCAAGCGGAAGAAAAGTGAACGGACACAGGGACTCAGCCACTGGGA
          E  R  R  A  L  I  K  R  K  K  S  E  R  T  G  T  Q  P  L  G   139
721   GTGCAGGGGCTGACAGAGGAGCAGCGGATGATGATCAGGGAGCTGATGGACGCTCAGATG
          V  Q  G  L  T  E  E  Q  R  M  M  I  R  E  L  M  D  A  Q  M   159
781   AAAACCTTTGACACTACCTTCTCCCATTTCAAGAATTCCGGCTGCCAGGGTGCTTAGC
          K  T  F  D  T  T  F  S  H  F  K  N  F  R  L  P  G  V  L  S   179
841   AGTGGCTGCGAGTTGCCAGAGTCTCTGCAGGCCCCATCGAGGGAAGAAGCTGCCAAGTGG
          S  G  C  E  L  P  E  S  L  Q  A  P  S  R  E  E  A  A  K  W   199
901   AGCCAGGTCCGGAAAGATCTGTGCTCTTTGAAGGTCTCCTCTCTCCAGCTGCGGGGGGAGGAT
          S  Q  V  R  K  D  L  C  S  L  K  V  S  L  Q  L  R  G  E  D   219
961   GGCAGTGTCTGGAACTACAAACCCCCAGCCGACGGTGGCGGGAAAGAGATCTTCTCCCTG
          G  S  V  W  N  Y  K  P  P  A  D  S  G  G  K  E  I  F  S  L   239
```

FIG. 1A-1

```
1021 CTGCCCCACATGGCTGACATGTCAACCTACATGTTCAAAGGCATCATCAGCTTTGCCAAA
      L  P  H  M  A  D  M  S  T  Y  M  F  K  G  I  I  S  F  A  K   259

1081 GTCATCTCCTACTTCAGGGACTTGCCCATCGAGGACCAGATCTCCCTGCTGAAGGGGGCC
      V  I  S  Y  F  R  D  L  P  I  E  D  Q  I  S  L  L  K  G  A   279

1141 GCTTTCGAGCTGTGTCAACTGAGATTCAACACAGTGTTCAACGCGGAGACTGGAACCTGG
      A  F  E  L  C  Q  L  R  F  N  T  V  F  N  A  E  T  G  T  W   299

1201 GAGTGTGGCCGGCTGTCCTACTGCTTGGAAGACACTGCAGGTGGCTTCCAGCAACTTCTA
      E  C  G  R  L  S  Y  C  L  E  D  T  A  G  G  F  Q  Q  L  L   319

1261 CTGGAGCCCATGCTGAAATTCCACTACATGCTGAAGAAGCTGCAGCTGCATGAGGAGGAG
      L  E  P  M  L  K  F  H  Y  M  L  K  K  L  Q  L  H  E  E  E   339

1321 TATGTGCTGATGCAGGCCATCTCCCTCTTCTCTCCCGACAGGCCCGGCGTGCTGCAGCAC
      Y  V  L  M  Q  A  I  S  L  F  S  P  D  R  P  G  V  L  Q  H   359

1381 CGCGTGGTGGACCAGCTGCAGGAGCAATTCGCCATTACTCTGAAGTCCTACATTGAATGC
      R  V  V  D  Q  L  Q  E  Q  F  A  I  T  L  K  S  Y  I  E  C   379

1441 AATCGGCCCCAGCCTGCTCATAGGTTCTTGTTCCTGAAGATCATGGCTATGCTCACCGAG
      N  R  P  Q  P  A  H  R  F  L  F  L  K  I  M  A  M  L  T  E   399

1501 CTCCGCAGCATCAATGCTCAGCACCACCAGGGCTGCTGGCCATCCAGGACATACACCCC
      L  R  S  I  N  A  Q  H  T  Q  R  L  L  R  I  Q  D  I  H  P   419

1561 TTTGCTACGCCCCCTCATGCAGGAGTTGTTCGGCATCACAGGTAGCTGAGCGGCTGCCCTT
      F  A  T  P  L  M  Q  E  L  F  G  I  T  G  S  *            434

1621 GGGTGACACCTCCGAGAGGCAGCCCAGAGCCCTGCTGGCCTGTCTCCCTAGGGCCA
1681 AGACAGATGGACACTGCCAAGAGCCGACAATGCCGACACCTCCCCACCCCCAGT
1741 CCTGCTATGACAGCTGGCTAGCATTCCTCAGGAGGACATGGGTGCCCCCCACCCCAGT
1801 TCAGTCTGTAGGAGTGAAGCCACAGACTCTTACGTGGAGAGTGCACTGACCTGTAGGTC
1861 AGGACCATCAGAGAGGCAAGGTTGCCCTTTCCTTTAAAAGGCCCTGTGGTCTGGGGAGA
1921 AATCCCTCAGATCCCACTAAAGTGTCAAGTGTGGAAGGGACCAAGGGACCAAGGATAGG
1981 CCATCTGGGGTCTATGCCCACATACCCACTTCCCACTTCCCTCCTCCTCTTTTCATTGCT
2041 ACCTCTAATAGTCCTGTCTCCCCACTTCCCACTTCCCTCCTCCTCTTCCGAGCTGCTTTG
2101 TGGGCTCCAGGCCTGACTACTCATCGGCGAGTGCATGAGTGCATGAGTATCTGTGG
```

FIG. 1A-2
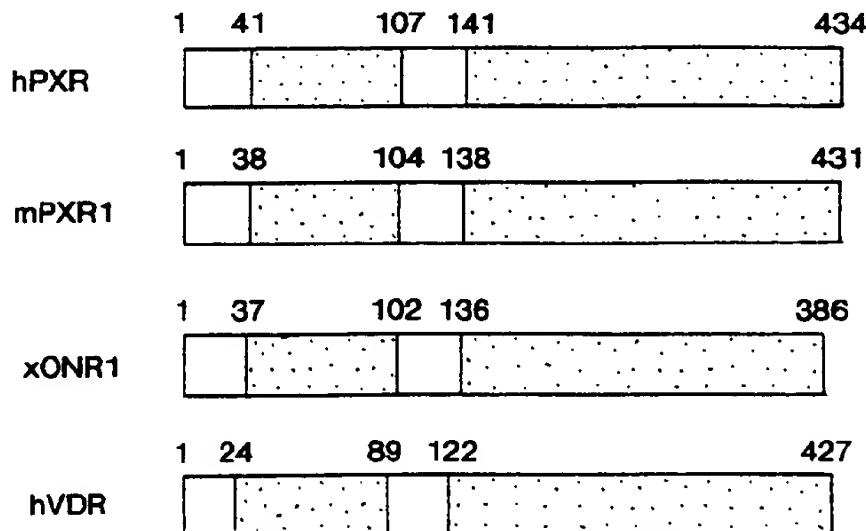
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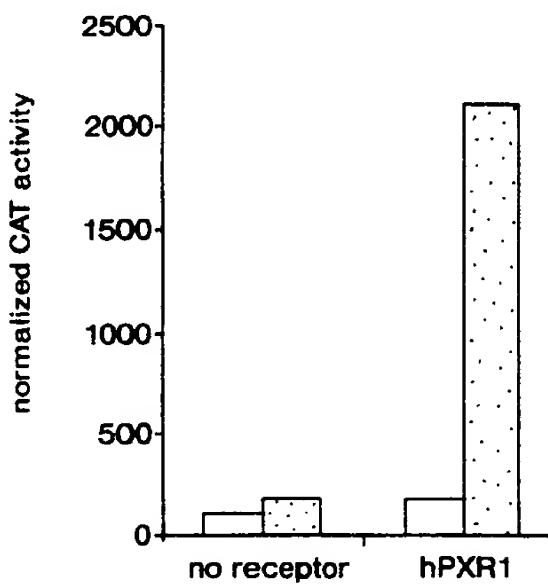
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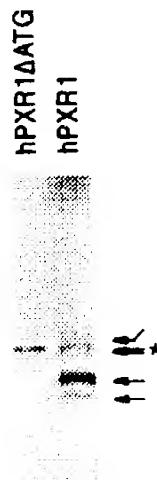
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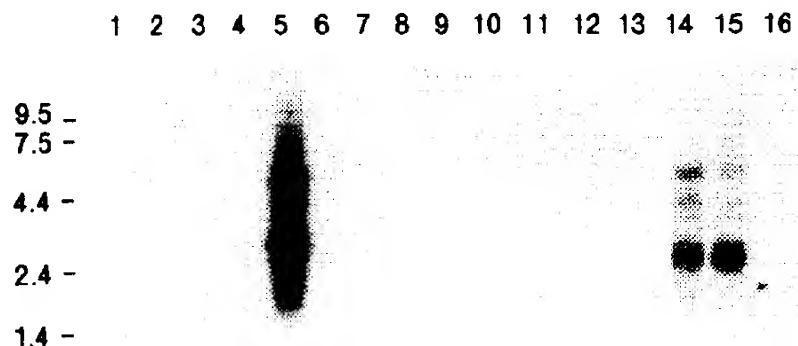
FIG. 2
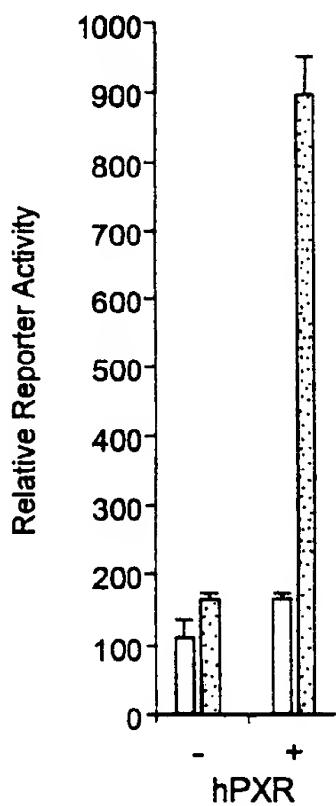
FIG. 3A

```
CYP3A4 IR6        ata  TGAACT  caaagg  AGGTCA  gtg
                       <-----          ----->

CYP3A4 IR6 m1     ata  TGTTCT  caaagg  AGAACA  gtg
                       <-XX--          --XX->

CYP3A4 IR6 m2     ata  ACAACT  caaagg  AGGTCA  gtg
                       XX----          ----->

CYP3A1 DR3        aga  TGAACT  tca  TGAACT  gtc
                       <-----       <-----
```

FIG. 3B

FIG. 3C

FIG. 4A

FIG. 4B

trans-nonachlor

lovastatin

clotrimazole

RU486

phenobarbital

rifampicin

dexamethasone-t-butylacetate

1   2   3   4

hPXR

mPXR1

FIG. 4C

Crabtree catalyst, dichloromethane
Tritium gas

Titanium chloride,
tetraethyl methylenediphosphonate,
methyl morpholine

[³H]GW-485801

SUBSTITUTE SHEET (RULE 26)
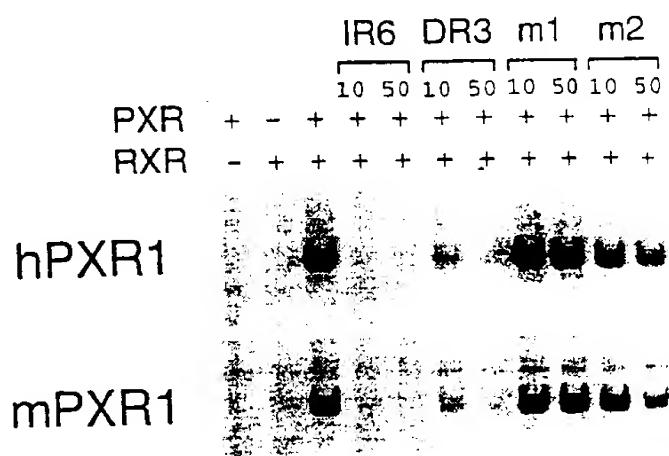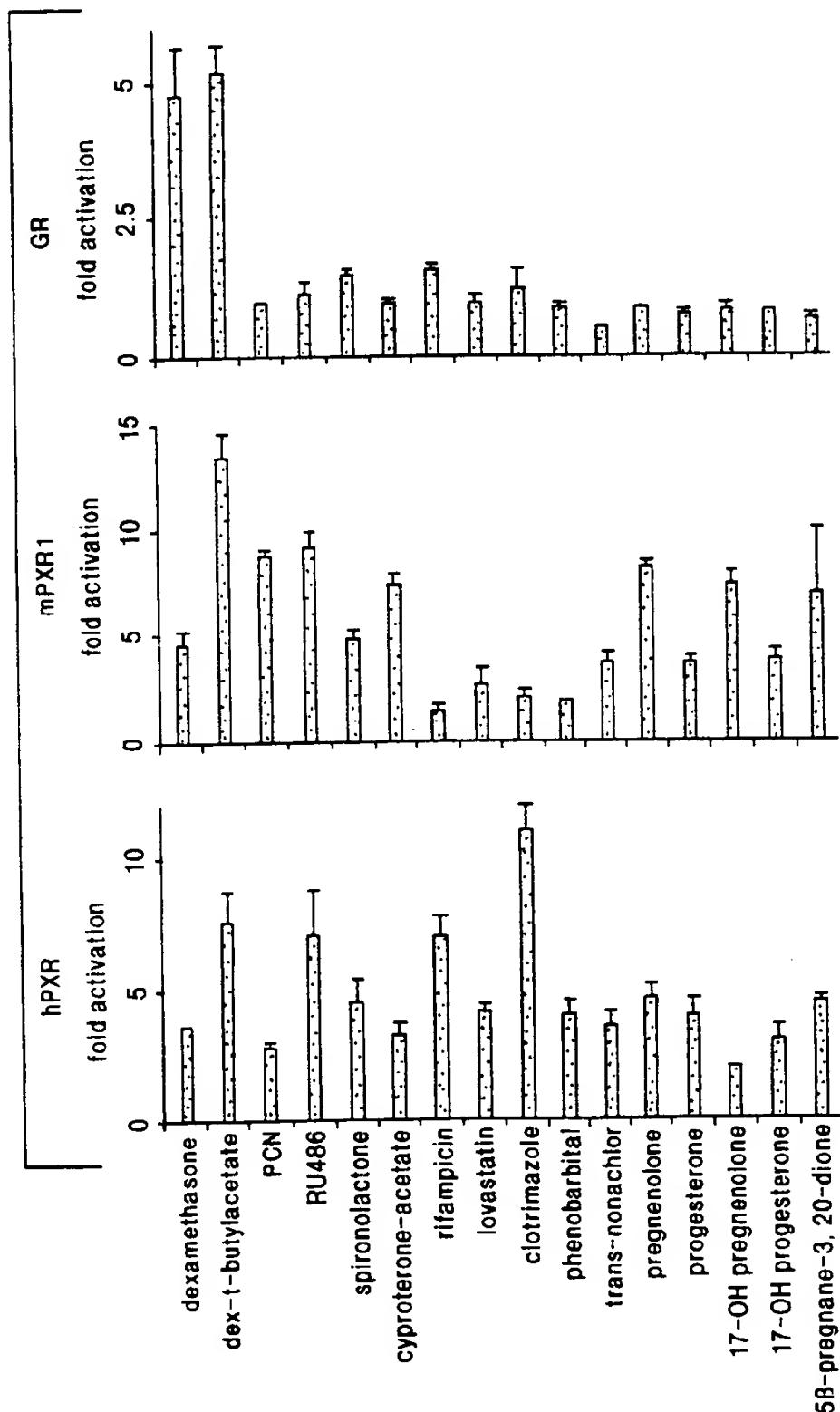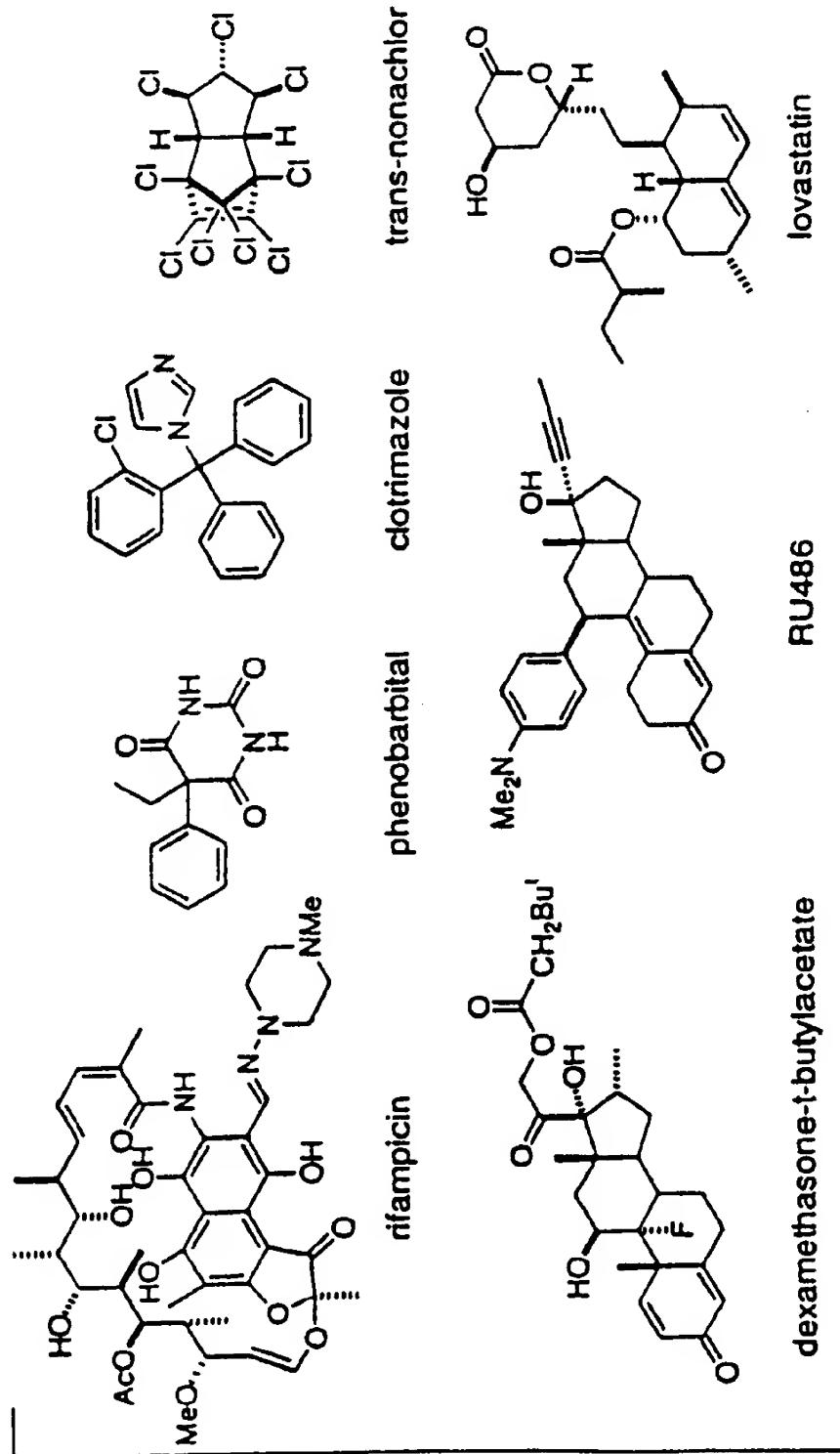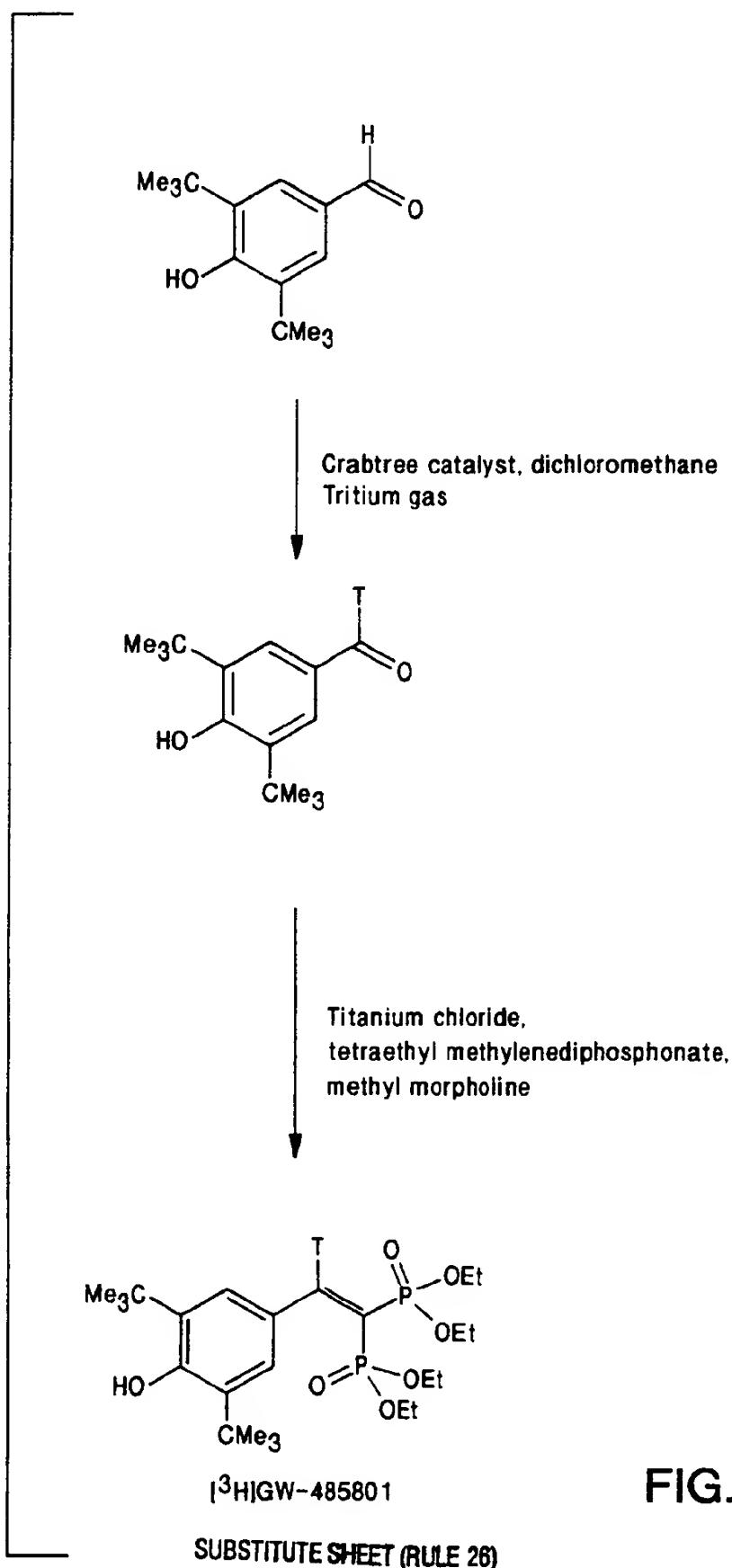
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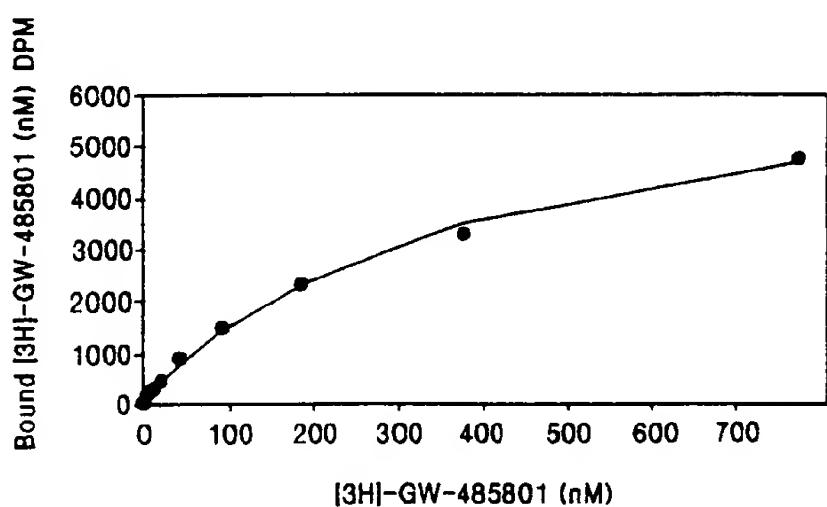
FIG. 6

SEQUENCE LISTING

<110> KLIEWER, Steven A.
      JONES, Stacey A.
      WILLSON, Timothy M.

<120> AN ORPHAN NUCLEAR RECEPTOR

<130> 510-125

<140> Unknown
<141> Unknown

<150> 60/079,593
<151> 1998-03-27

<160> 14

<170> PatentIn Ver. 2.0

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  1               5                   10
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<211> 316
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Protein

<400> 11

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1               5                       10                      15

Thr Gln Pro Leu Gly Val   Gln Gly Leu Thr Glu Glu Gln Arg Met Met
            20                        25                      30

Ile Arg Glu Leu Met Asp   Ala Gln Met Lys Thr Phe Asp Thr Thr Phe
        35                        40                      45

Ser His Phe Lys Asn Phe   Arg Leu Pro Gly Val Leu Ser Ser Gly Cys
        50              55                  60

Glu Leu Pro Glu Ser Leu   Gln Ala Pro Ser Arg Glu Glu Ala Ala Lys
65                  70                    75                      80

Trp Ser Gln Val Arg Lys   Asp Leu Cys Ser Leu Lys Val Ser Leu Gln
                85                        90                      95

Leu Arg Gly Glu Asp Gly   Ser Val Trp Asn Tyr Lys Pro Pro Ala Asp
            100                       105                     110

Ser Gly Gly Lys Glu Ile   Phe Ser Leu Leu Pro His Met Ala Asp Met
            115                   120                     125

Ser Thr Tyr Met Phe Lys   Gly Ile Ile Ser Phe Ala Lys Val Ile Ser
        130               135                 140

Tyr Phe Arg Asp Leu Pro   Ile Glu Asp Gln Ile Ser Leu Leu Lys Gly
145                 150                  155                     160

Ala Ala Phe Glu Leu Cys   Gln Leu Arg Phe Asn Thr Val Phe Asn Ala
            165                       170                     175

Glu Thr Gly Thr Trp Gl    Cys Gly Arg Leu Ser Tyr Cys Leu Glu Asp
            180                   185                     190

Thr Ala Gly Gly Phe Gl    Gln Leu Leu Leu Glu Pro Met Leu Lys Phe
        195                   200                     205

His Tyr Met Leu Lys Ly    Leu Gln Leu His Glu Glu Glu Tyr Val Leu
        210               215                 220

Met Gln Ala Ile Ser Le    Phe Ser Pro Asp Arg Pro Gly Val Leu Gln
225             2                         235                     240

His Arg Val Val Asp Gl     Leu Gln Glu Gln Phe Ala Ile Thr Leu Lys
            245                       250                     255

Ser Tyr Ile Glu Cys A      Arg Pro Gln Pro Ala His Arg Phe Leu Phe
            260                       265                     270

Leu Lys Ile Met Ala M      Leu Thr Glu Leu Arg Ser Ile Asn Ala Gln
            275                       280                     285
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His Thr Gln Arg Leu Leu Arg Ile Gln Asp Ile His Pro Phe Ala Thr
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Pro Leu Met Gln Glu Leu Phe Gly Ile Thr Gly Ser
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<211> 262
<212> PRT
<213> Artificial Sequence

<220>
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Glu Ala Glu Leu Ala Val Glu Pro Lys Thr Glu Thr Tyr Val Glu Ala
                20              25              30

Asn Met Gly Leu Asn Pro Ser Ser Pro Asn Asp Pro Val Thr Asn Ile
            35              40              45

Cys Gln Ala Ala Asp Lys Gln Leu Phe Thr Leu Val Glu Trp Ala Lys
    50              55              60

Arg Ile Pro His Phe Ser Glu Leu Pro Leu Asp Asp Gln Val Ile Leu
65              70              75              80

Leu Arg Ala Gly Trp Asn Glu Leu Leu Ile Ala Ser Phe Ser His Arg
                85              90              95

Ser Ile Ala Val Lys Asp Gly Ile Leu Leu Ala Thr Gly Leu His Val
            100             105             110

His Arg Asn Ser Ala His Ser Ala Gly Val Gly Ala Ile Phe Asp Arg
        115             120             125

Val Leu Thr Glu Leu Val Ser Lys Met Arg Asp Met Gln Met Asp Lys
    130             135             140

Thr Glu Leu Gly Cys Leu Arg Ala Ile Val Leu Phe Asn Pro Asp Ser
145             150             155             160

Lys Gly Leu Ser Asn Pro Ala Glu Val Glu Ala Leu Arg Glu Lys Val
            165             170             175

Tyr Ala Ser Leu Glu Ala Tyr Cys Lys His Lys Tyr Pro Glu Gln Pro
        180             185             190

Gly Arg Phe Ala Lys Leu Leu Leu Arg Leu Pro Ala Leu Arg Ser Ile
        195             200             205

Gly Leu Lys Cys Leu Glu His Leu Phe Phe Phe Lys Leu Ile Gly Asp
    210             215             220

Thr Pro Ile Asp Thr Phe Leu Met Glu Met Leu Glu Ala Pro His Gln
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Met Thr
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<211> 2146
<212> DNA
<213> Artificial Sequence

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cggctccttg gtaaagctac tccttgatcg atcctttgca ccggattgtt caaagtggac 240
cccaggggag aagtcggagc aaagaactta ccaccaagca gtccaagagg cccagaagca 300
aacctggagg tgagacccaa agaaagctgg aaccatgctg actttgtaca ctgtgaggac 360
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ccatctgggg tctatgccca cataccacg tttgttcgct tcctgagtct tttcattgct 2040
acctctaata gtcctgtctc ccacttccca ctcgttcccc tcctcttccg agctgctttg 2100
tgggctccag gcctgtactc atcggcaggt gcatgagtat ctgtgg 2146

<210> 14
<211> 414
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Protein

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 1               5                  10                  15

Cys Glu Asp Thr Glu Ser Val Pro Gly Lys Pro Ser Val Asn Ala Asp
                20                  25                  30

Glu Glu Val Gly Gly Pro Gln Ile Cys Arg Val Cys Gly Asp Lys Ala
        35                  40                  45

Thr Gly Tyr His Phe Asn Val Met Thr Cys Glu Gly Cys Lys Gly Phe
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              50                      55                      60
Phe Arg Arg Ala Met Lys Arg Asn Ala Arg Leu Arg Cys Pro Phe Arg
65                  70                  75                  80

Lys Gly Ala Cys Glu Ile Thr Arg Lys Thr Arg Arg Gln Cys Gln Ala
                85                  90                  95

Cys Arg Leu Arg Lys Cys Leu Glu Ser Gly Met Lys Lys Glu Met Ile
            100                 105                 110

Met Ser Asp Glu Ala Val Glu Glu Arg Arg Ala Leu Ile Lys Arg Lys
        115                 120                 125

Lys Ser Glu Arg Thr Gly Thr Gln Pro Leu Gly Val Gln Gly Leu Thr
        130                 135                 140

Glu Glu Gln Arg Met Met Ile Arg Glu Leu Met Asp Ala Gln Met Lys
145                 150                 155                 160

Thr Phe Asp Thr Thr Phe Ser His Phe Lys Asn Phe Arg Leu Pro Gly
                165                 170                 175

Val Leu Ser Ser Gly Cys Glu Leu Pro Glu Ser Leu Gln Ala Pro Ser
            180                 185                 190

Arg Glu Glu Ala Ala Lys Trp Ser Gln Val Arg Lys Asp Leu Cys Ser
        195                 200                 205

Leu Lys Val Ser Leu Gln Leu Arg Gly Glu Asp Gly Ser Val Trp Asn
        210                 215                 220

Tyr Lys Pro Pro Ala Asp Ser Gly Gly Lys Glu Ile Phe Ser Leu Leu
225                 230                 235                 240

Pro His Met Ala Asp Met Ser Thr Tyr Met Phe Lys Gly Ile Ile Ser
                245                 250                 255

Phe Ala Lys Val Ile Ser Tyr Phe Arg Asp Leu Pro Ile Glu Asp Gln
            260                 265                 270

Ile Ser Leu Leu Lys Gly Ala Ala Phe Glu Leu Cys Gln Leu Arg Phe
        275                 280                 285

Asn Thr Val Phe Asn Ala Glu Thr Gly Thr Trp Glu Cys Gly Arg Leu
        290                 295                 300

Ser Tyr Cys Leu Glu Asp Thr Ala Gly Gly Phe Gln Gln Leu Leu Leu
305                 310                 315                 320

Glu Pro Met Leu Lys Phe His Tyr Met Leu Lys Lys Leu Gln Leu His
                325                 330                 335

Glu Glu Glu Tyr Val Leu Met Gln Ala Ile Ser Leu Phe Ser Pro Asp
                340                 345                 350

Arg Pro Gly Val Leu Gln His Arg Val Val Asp Gln Leu Gln Glu Gln
            355                 360                 365

Phe Ala Ile Thr Leu Lys Ser Tyr Ile Glu Cys Asn Arg Pro Gln Pro
        370                 375                 380

Ala His Arg Phe Leu Phe Leu Lys Ile Met Ala Met Leu Thr Glu Phe
385                 390                 395                 400
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Ala Thr Pro Leu Met Gln Glu Leu Phe Gly Ile Thr Gly Ser
              405   410

	International application No.
	PCT/US99/06737

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :Please See Extra Sheet.
US CL   :435/7.1, 69.1, 320.1, 325; 530/350; 536/23.4, 23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/7.1, 69.1, 320.1, 325; 530/350; 536/23.4, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ----- A, P	BERTILSSON et al. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proc. Natl. Acad. Sci. USA. October 1998, Vol. 95, pages 12208-12213, especially page 12208 and figures 1-5.	1-15, 17-20, 23 --------- 16, 21-22, 24

☐   Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"B"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 AUGUST 1999	11 AUG 1999

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized Officer MICHAEL PAK Telephone No. (703) 308-1234

Form PCT/ISA/210 (second sheet)(July 1992)★

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 14/00, 14/435, 14/705; C12N 5/16, 15/12, 15/62, 15/63; G01N 33/53, 33/566

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, BIOSCIENCE, REGISTRY, CAPLUS, MEDLINE, GENESEQ, EST, PIR
search terms: nuclear receptor, PXR, cytochrome P-450 monooxygenase, GW-485801

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-10, drawn to human nuclear receptor, nucleic acid encoding the said receptor, vector and a host cell containing the said DNA, a method of making the receptor, and a method of screening for test compound using the receptor, first use.
Group II, claim(s) 11, drawn to a method for screening a test compound using a transformed cell.
Group III, claim(s) 12, drawn to a compound which induces CYP3A4.
Group IV, claim(s)13-19, drawn to a compound that activates hpXR, and a method of their use.
Group V, claim(s) 20-22, drawn to a method of associating a particular disease or condition with modulation of PXR.
Group VI, claim(s) 23 and 24, drawn to a method of preventing or treating a disease or condition.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of the invention of Group I is the human nuclear receptor protein which differs from transformed cell of Group II, and the compounds of Groups III and IV. Although the special technical feature of Groups I, V and VI is the same, the invention of Groups V and VI represent a second and a third use for the protein. Also, the special technical feature of the invention of Group II, i.e., the transformed cell is different from the compounds of Groups III and IV, and the receptor protein of Groups V and VI. The compounds of Group III appear to have different biological effect from those of Group IV and differ from the protein of Group V and VI. In addition, the special technical feature of the invention of Group IV, i.e., the compounds that activate hPXR, differ from the protein of Groups V and VI. Thus, the inventions of Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1.